Involvement of active gelatinase B/matrix metalloproteinase-9 in vitreous hemorrhagic transformation of proliferative diabetic retinopathy

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Abstract

Objective: To investigate the presence and activation status of matrix metalloproteinase (MMP)-2 and MMP-9 in vitreous samples from proliferative diabetic retinopathy (PDR) patients.

Research Design and Methods: Vitreous samples were obtained from 132 non-diabetic and diabetic patients undergoing vitrectomy for the treatment of rhegmatogenous retinal detachment (RD) and PDR. PDR patients were diagnosed for the presence of hemorrhage and/or patent new vessels. Quantitative assays were performed for vitreous protein content, MMP-2, MMP-9, TIMP-1 and hemoglobin. Qualitative evaluation of the MMP-2 and MMP-9 activation status was performed by zymography.

Results: Vitreous samples prominently contained proMMP-2 but levels were unselectively related to total protein content. ProMMP-9 and activated MMP-9 levels were significantly increased in PDR patients. TIMP-1 levels were significantly elevated in PDR patients and functionally inhibited activation of MMP-9 in vitreous samples. None of the parameters significantly differed between PDR patients with new vessels and those with inactive disease. However, activated MMP-9 levels in vitreous samples of PDR patients with hemorrhage (75.7 ± 106.3 scanning units per 2 µl) were significantly higher than those in PDR patients without hemorrhage (7.1 ± 16.2 scanning units per 2 µl) ($p < 0.001$; Mann-Whitney test) and strongly correlated with hemoglobin levels ($r = 0.7525$; $p < 0.001$). Serum did not contain activated MMP-9.

Conclusions: We conclude that activated MMP-9 in the vitreous of PDR patients with vitreous hemorrhagic transformation may be involved in the hemorrhagic process at the blood-vitreous interface, in ischemic neovessels.

***Key words*: proliferative diabetic retinopathy, neovascularization, metalloproteinases, vitreous, hemorrhage.**

Proliferative diabetic retinopathy (PDR) is a microangiopathic complication of diabetes involving the retinal microvasculature. Similar to nephropathy and neuropathy as other long-term complications of diabetes, the evolution of PDR correlates well with the severity and duration of hyperglycaemia (1). Progression of microangiopathy may be explained by an increased metabolic flux through the polyol pathway, the generation of advanced glycation end-products and inadequate regulation of the protein kinase C activity. These mechanisms together induce thickening of retinal capillary basement membranes and narrowing of the microvascular lumen, which in turn induces occlusive angiopathy, tissue hypoxia and ischemia. Weakening of the retinal capillaries induces focal dilatations (microaneurysms). When the wall of a capillary or microaneurysm is sufficiently weakened, it may give rise to intraretinal haemorrhages, also denominated dot-blot haemorrhages. The proliferative stage of PDR develops from ensuing progressive ischemia with concomitant cellular alterations, e.g. the release of growth factors that induce angiogenesis. Vascular endothelial growth factor (VEGF) is a dominant inducer of retinal neovascularization (2-5).

Retinal neovascularization typically involves the growth of neovessels from the venous side of the retinal circulation into the vitreous. These neovessels are prone to bleeding (vitreous hemorrhage), which may result in profound loss of vision when the macula is obscured.

Diabetes is associated with abnormal angiogenesis, both impaired and/or excessive (6,7). Neovascularization involves proteolytic degradation of the venules' basement membrane to facilitate endothelial cell release and thereupon, migration of endothelial cells into the perivascular stroma, again promoted by proteolysis of extracellular matrix components. Degradation of extracellular matrix components in either normal physiological and pathological conditions is accomplished largely through the controlled action of matrix metalloproteinases (MMPs) (8-10). Recent reports have suggested a decrease in renal (11) and vascular tissue MMP-9 (12) and an increase in endothelial cell and macrophage MMP-9 in response to glucose (13). With regard to retinal neovascularization, active forms of two particular MMP members, MMP-2 and MMP-9 (respectively gelatinase A and gelatinase B) were shown in epiretinal neovascular membranes from patients with PDR (14). Extraretinal neovascularization after ischemia-induced retinal neovascularization was diminished in mice treated with the overall hydroxamate MMP-inhibitor batimastat (15) and in *MMP-2*$^{-/-}$ knockout mice (16).

Soluble MMPs are produced as inactive precursors and are processed into active enzymes by proteolytic removal of an amino-terminal pro-peptide upon secretion into the extracellular space. Activation of MMP zymogens involves disruption of a coordination bond formed between a highly conserved unpaired cysteine in the amino-terminal pro-peptide of the pro-MMP molecule and the zinc ion at the active center (17). After secretion and activation, MMP activities can be downregulated by inhibition or (auto)-degradation. Tissue inhibitors of metalloproteinases (TIMPs) are specific endogenous inhibitors that bind to individual MMPs with high affinity. MMP-9 (gelatinase B) activation is a final event in a broader MMP proteolytic cascade and as a consequence, MMP-9 activity is controlled at more than one level by TIMP-1. TIMP-1 first inhibits the activation of MMP-9 by binding to other MMP-members that are MMP-9-activators. Once MMP-9 is activated, TIMP-1 may directly block MMP-9 activity.

We investigated in this study the presence and activation status of MMP-2 and MMP-9 in vitreous samples of patients with PDR and rhegmatogenous retinal detachment (RD).

Methods

Patients

Vitreous samples were obtained from 132 consecutive patients (132 eyes) undergoing vitrectomy for the treatment of rhegmatogenous retinal detachment without proliferative vitreoretinopathy (RD) (65 specimens) and proliferative diabetic retinopathy (PDR) (67 specimens). All surgeries were performed by one of the authors (AMA). The indications for vitrectomy in eyes with RD were giant breaks, macular breaks, and bullous retinal detachment with complex arrangement of breaks. The indications for vitrectomy in eyes with PDR were tractional retinal detachment, and/or nonclearing vitreous hemorrhage. In patients with PDR the clinical ocular findings were graded at the time of vitrectomy for the presence of hemorrhage, tractional retinal detachment, and presence or absence of patent new vessels on the retina or optic disc. Patients with active PDR were graded as such on the basis of visible patent new vessels on the retina or optic disc or their absence (inactive PDR). Tractional retinal detachment was present in 43 patients, and vitreous hemorrhage in 34 patients. Active PDR was present in 24 patients. The study was conducted according to the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients.

Sampling

The samples were collected undiluted by manual suction into a syringe through the aspiration line of the vitrectomy, before opening the infusion line. The samples were centrifuged, and the supernatants were frozen at -40°C until they were assayed.

Biochemical analyses

Total vitreous protein content was determined according to Bradford (18). Sandwich ELISA for total MMP-9 was performed with a monoclonal capture antibody REGA-3G12 (19) and a polyclonal detection antibody (R&D Systems, Abingdon, United Kingdom). Sandwich ELISA for TIMP-1 was performed with a Duoset® ELISA kit (R&D Systems). For semi-quantitative zymography analysis (20), 2 µl undiluted vitreous fluid was loaded on 7.5% acrylamide gels with 0.1% co-polymerised gelatin and processed as previously described (**Fig. 1**). Densitometry analysis was performed with the use of the ImageMaster 1D Elite software (Pharmacia Biotech, Uppsala, Sweden). In order to evaluate the effectiveness of quantitative MMP-9 assessments in vitreous samples, a dilution series of pure neutrophil MMP-9 was added to vitreous samples, which were subsequently subjected to zymography analysis (with subsequent densitometry analysis of zymolytic bands) and ELISA. Zymography analysis, which is performed under denaturing conditions, was shown to be superior to (native) ELISA as a quantitative MMP-9-assay for vitreous samples, probably because MMP-9 forms complexes in the vitreous, e.g. with TIMP-1 and/or collagen, which may interfere with the binding to antibodies. Therefore, we chose for zymography analysis to quantitatively determine MMP-9 (and MMP-2) levels in vitreous samples.

Hemoglobin concentrations were determined with the use of a modified method from Crosby and Furth (21). In advance, a hemoglobin standard was prepared from healthy donor blood that was collected in heparinized tubes. Red blood cells were washed three times in phosphate-buffered saline and hemolysis was induced by hypotonic shock in cold bi-destilled H_2O. Cell debris was peletted via centrifugation at 3000 g and hemoglobin in the supernatant was calculated based on the molar extinction coefficient at 415 nm (A_{415nm} = 130470 M^{-1} cm^{-1}). This solution with known hemoglobin concentration was further used as a standard. Importantly, additions of albumin (30 mg/ml) did not interfere with the assay. For sensitive hemoglobin quantification in vitreous samples (21), 200 µl of tetramethyl benzidine (TMB) working solution (1 mg TMB in 90:10 acetic acid: H_2O solution) and 100 µl H_2O_2 working solution (1% H_2O_2 in bi-destilled H_2O) was added to 5 µl vitreous sample or standard. The optical density at 450 nm was determined after 10 min incubation at room temperature. Absolute hemoglobin levels were calculated by relation of test samples with the standard line analysis.

Statistical methods

Data were analysed by the chi-square test, the Mann-Whitney test, and correlation analysis. The chi-square test was used to compare the percentages of vitreous samples with detectable levels of MMP-9, and activated MMP-9, from eyes with RD and PDR. The Mann-Whitney test was used to compare means for two independent groups. Correlation analysis was conducted to investigate the strength of the linear relationships between the variables under investigation. A p-value less than 0.05 indicated statistical significance.

Results

The mean total protein level in the vitreous of patients with PDR (5.6 ± 4.0 mg/ml) was significantly higher than that in patients with RD (3.97 ± 7.1 mg/ml).

All vitreous samples prominently contained proMMP-2; as determined by ELISA. The mean proMMP-2 level in patients with PDR (685.8 ± 222.3 scanning units per 2 µl) was significantly higher than that in patients with RD (599.4 ± 261.2 scanning per 2 µl) (p=0.0264; Mann-Whitney test). However, proMMP-2 levels correlated with vitreous protein levels both in RD (r = 0.5052; p < 0.001) and PDR patients (r = 0.4951; p < 0.001) (**Fig. 1, left panels**). If only patients with vitreous protein concentrations in the same range (between 1.2 mg/ml and 19 mg/ml) were taken into account, mean proMMP-2 levels were similar between RD (690.6 ±, 207.9 scanning units per 2 µl) and PDR patients (702.9 ± 220.7 scanning units per 2 µl).

Activated MMP-2 was only marginally detected (on zymograms) in comparison with proMMP-2 and did not correlate with a particular disease state (Fig. 2).

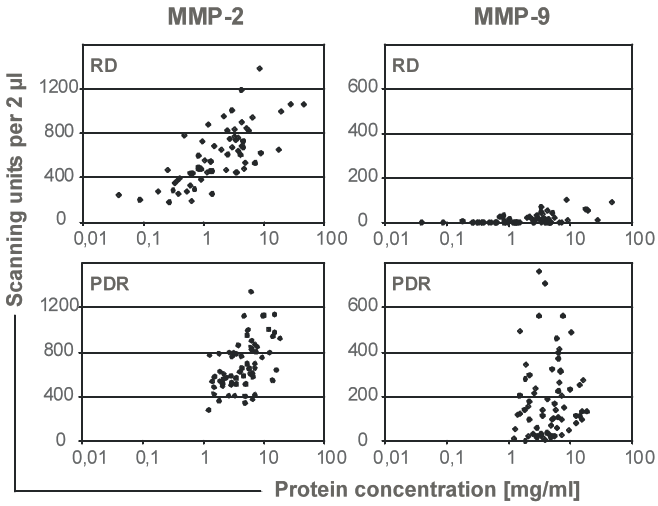

Figure 1: Relative vitreous MMP-2 and MMP-9 levels (in terms of scanning units per 2 µl) in function of total vitreous protein content in RD and PDR patients.

ProMMP-9 was detected in 24 (36.9%) of 65 vitreous samples from eyes with RD and in 66 (98.5%) of 67 vitreous samples from eyes with PDR (**Fig. 2**), and the difference between the two percentages was statistically significant (p < 0.001; Chi-square test). The mean proMMP-9 level in patients with PDR (187.5 ± 173.4 scanning units per 2 µl) was significantly higher than that in patients with RD (12.9 ± 22.6 scanning units per 2 µl) (p < 0.001; Mann-Whitney test). **Fig 1 (right panels)** illustrates that upregulation of proMMP-9 in PDR patients did not correlate with total vitreous protein levels.

Activated (82 kDa) MMP-9 was detected in 4 (6.2%) of 65 vitreous samples from eyes with RD, compared to 36 (53.7%) of 67 vitreous samples from eyes with PDR, and the difference between the two percentages was statistically significant (p < 0.001; Chi-square test). The mean activated MMP-9 level in the vitreous of patients with PDR (43.9 ± 85.4 scanning units per 2 µl) was significantly higher than that in patients with RD (2.4 ± 12.1 scanning units per 2 µl) (p < 0.001; Mann-Whitney test). Activated MMP-9 levels did not correlate with vitreous protein levels (r = 0.0760; p = 0.3536).

The mean TIMP-1 level in the vitreous of patients with PDR (468 ± 422.2 ng/ml) was significantly higher than that in patients with RD (308.8 ± 299.6 ng/ml) (p = 0.004, Mann-whitney test). Conversion of MMP-9 scanning units to absolute MMP-9 concentrations through standardization of densitometry analysis revealed that TIMP-1/MMP-9 ratios in the vitreous were systematically in excess of 1 (up to 5).

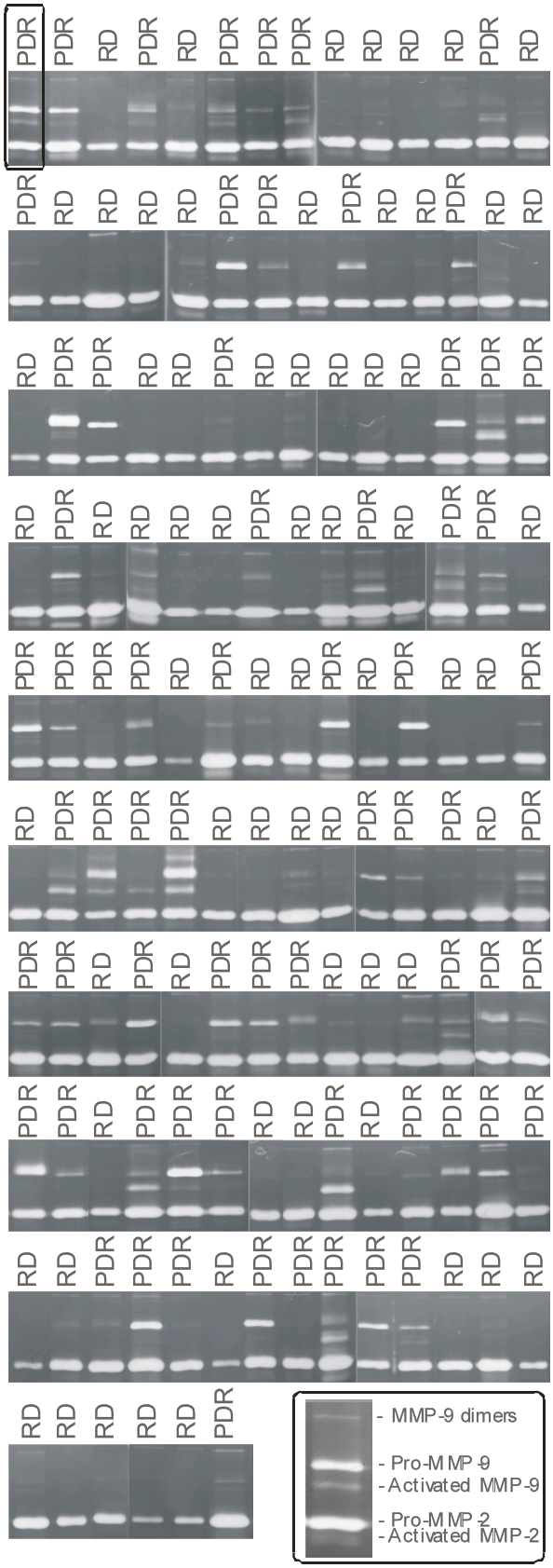

Figure 2: Zymography analysis of randomly sorted RD (n = 65) and PDR (n = 67) patients. A two-fold magnification of the boxed sample is included as a legend with designation of the respective MMP forms.

ProMMP-9 levels in PDR patients did not correlate with activated MMP-9 levels ($r = -0.1005$; $p = 0.4183$). Therefore, we hypothesized that proMMP-9 and activated MMP-9 may be independently linked to discrete subpopulations of PDR patients. We have subcategorized PDR as a) active or inactive PDR on the basis of visible patent new vessels on the retina or optic disc and b) PDR complicated by vitreous hemorrhagic transformation or not. The mean proMMP-2, proMMP-9 and activated MMP-9 fractions in patients with active PDR did not significantly differ from those with inactive PDR. Also, the mean proMMP-2 level and proMMP-9 level in PDR patients with hemorrhage did not significantly differ from those without hemorrhage. However, the mean activated MMP-9 level in PDR patients with hemorrhage (75.7 ± 106.3 scanning units per 2 µl) was significantly higher than that in PDR patients without hemorrhage (7.1 ± 16.2 scanning units per 2 µl) ($p < 0.001$; Mann-Whitney test). Thereupon, hemoglobin was titrated as a measure for the amount of erupted blood. We found a strong positive correlation between activated MMP-9 levels and hemoglobin levels in the entire study group ($r = 0.778$; $p < 0.001$) and in the PDR group of patients ($r = 0.7525$; $p < 0.001$). Hemoglobin levels did not correlate with proMMP-9 levels in PDR patients($r = 0.098$) and in the entire study group ($r = 0.1005$; $p = 0.2225$) (**Fig 3**).

Next, we studied whether the activation of proMMP-9 by its most prominent activator MMP-3 might take place in the vitreous and how this process might be controlled by TIMP-1. The vitreous TIMP-1/MMP-9 ratio was largely in excess of 1, particularly in PDR patients, which is a setting that counterbalances proMMP-9 activation. Incubation of vitreous fluid from selected patients in the presence of MMP-3 (as prominent MMP-9 activator) indeed revealed a 'buffering capacity' of the vitreous with regard to MMP-9 activation. The minimal MMP-3 level that was needed to achieve activation in vitreous samples shifted to high levels in comparison with a TIMP-1-free recombinant MMP-9 control preparation. The size of the shift was dependent on the vitreous TIMP-1 concentration (**Fig. 4**). Thus, MMP-9 activation did probably not occur in the vitreous. In addition, activated MMP-9 could not originate from serum since no activated MMP-9 was detected in serum samples. Data of quantitative assessments of MMP-9 in serum were ambiguous since degranulation of neutrophils had occurred during sample collection. As a result, serum samples contained considerable amounts of 'irrelevant' proMMP-9. Anyhow, no 82 kDa zymolytic band could be detected (data not shown).

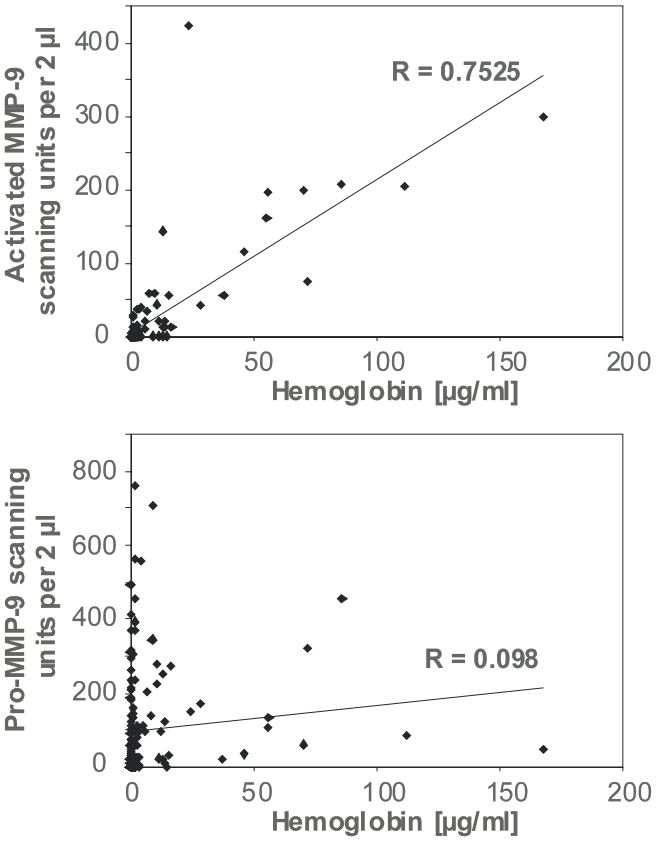

Figure 3: Vitreous proMMP-9 and activated MMP-9 levels in function of vitreous hemoglobin levels in PDR patients.

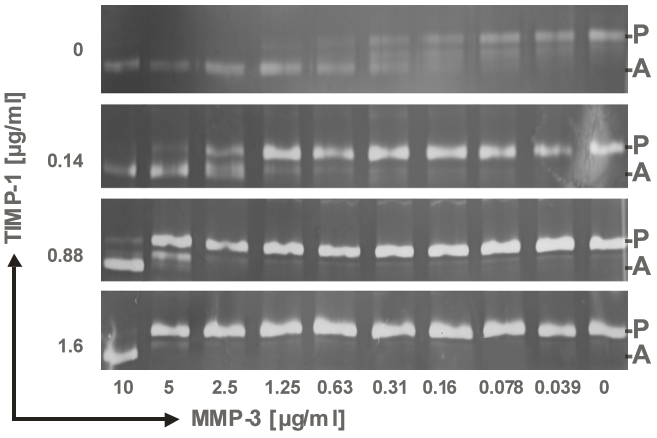

Figure 4: Buffering capacity of vitreous with regard to proMMP-9 activation. Control TIMP-1-free recombinant proMMP-9 (upper panel) and selected proMMP-9-containing vitreous samples with variable endogenous TIMP-1 concentrations (lower three panels) were incubated with a dilution series of activated MMP-3. A TIMP-1 dependent buffering capacity of the vitreous with regard to proMMP-9 activation can be deduced from the shift in the amount of MMP-3 that is needed to achieve proMMP-9 activation.

Discussion

Matrix metalloproteinases MMP-2 and MMP-9 have previously been implicated in neovascularization processes, including PDR (2-5,14-16,22,23). Our results indicate that vitreous fluid is not really suitable as sample to view retinal neovascularization. MMP-2 levels are not elevated in patients with PDR and the activation status is low. MMP-9 levels are significantly elevated in PDR patients but are not extra pronounced in active PDR in comparison with inactive PDR. Yet, the 'neovascularization status' of endothelial cells is characterized by upregulation and activation of gelatinases, as previously described in *in vitro* settings (24) and in fibrovascular membranes of PDR patients (25). Neovascularization can probably not be detected in the vitreous.

A more important novel finding in this study was the discovery of activated MMP-9 in the vitreous of PDR patients and the link with the presence and degree of vitreous hemorrhage. Levels of pro-MMP-9 were elevated in PDR patients but did neither correlate with vitreous hemorrhage or with activated MMP-9 levels. This may indicate that the presence of the respective forms is related to disparate processes, and so that activated MMP-9 was not processed from resident proMMP-9 in the vitreous. It is indeed unlikely that activation of proMMP-9 took place in the vitreous, regarding the high vitreous TIMP-1 levels, which imply principal inhibition of the MMP-9 activation cascade (26,27). Activated MMP-9 neither originated from serum, since we did not detect any activated MMP-9 in sera. Moreover, activation of proMMP-9 is unlikely occurring in serum due to high TIMP-1 levels and presence of the broad-spectrum protease inhibitor α_2-macroglobulin.

Per exclusionem, we favor the possibility that activated MMP-9 was generated in a process that occurred in the layer between the microvascular lumen and the vitreous and suggest that the observed activated MMP-9 was processed from proMMP-9 released by microvascular endothelial cells, probably at the basolateral side, in response to an ischemic state. MMP-9 activity may then have degraded the basal membrane. A gap within the basal membrane easily causes leakage of blood and meanwhile the seeping of activated MMP-9 itself. Activated MMP-9 is not often detected in biological fluids because it is quickly cleared by autocatalysis. In the present study of vitreous samples, autocatalysis of gelatinase B was probably prevented because of high TIMP-1 levels. Finally, it is interesting to note that this prospective mechanism for hemorrhagic transformation of ischemic microvessels may also be valid for dot-blot

hemorrhages, which occur intra-retinally in the pre-proliferative stage of PDR.

Activation of proMMP-9 in ischemic microvessels may have occurred through the plasmin activation cascade. Plasmin has previously been reported to be involved in hemorrhagic transformation of cerebral ischemia, probably by catalysis of proMMP-9 activation (26,28-31). Diabetic patients are at higher risk to develop cerebral hemorrhage after tissue-plasminogen activator (t-PA) treatment, which turns plasminogen into plasmin (32). With relevance to retinal microvessels, systemic thrombolytic therapy with t-PA can lead to devastating intraocular hemorrhages (33). Endogenous levels of t-PA are elevated in patients with PDR. The presence of activated MMP-9 in PDR patients with vitreous hemorrhage may therefore indicate that mechanisms provoking hemorrhagic transformation in cerebral ischemia may be valid for retinal ischemia. We suggest that special attention be payed to this mechanism in diabetes patients.

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