VEGF Secreted by Hypoxic Müller Cells Induces MMP-2 Expression and Activity in Endothelial Cells to Promote Retinal Neovascularization in Proliferative Diabetic Retinopathy

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Abstract

In proliferative diabetic retinopathy (PDR), retinal ischemia promotes neovascularization (NV), which can lead to profound vision loss in diabetic patients. Treatment for PDR, panretinal photocoagulation, is inherently destructive and has significant visual consequences. Therapies targeting vascular endothelial growth factor (VEGF) have transformed the treatment of diabetic eye disease, but have proven inadequate for treating NV, prompting exploration for additional therapeutic options for PDR patients. In this regard, extracellular proteolysis is an early and sustained activity strictly required for NV. Extracellular proteolysis in NV is facilitated by the dysregulated activity of matrix metalloproteinases (MMPs). Here we set out to better understand the regulation of MMPs by ischemia in PDR. We demonstrate that accumulation of hypoxia-inducible factor-1α in Müller cells induces expression of VEGF, which in turn, promotes increased MMP-2 expression and activity in neighboring endothelial cells. MMP-2 expression was detected in endothelial cells in retinal NV tissue from PDR patients while MMP-2 protein levels were elevated in the aqueous of PDR patients compared to controls. Our findings demonstrate a complex interplay among hypoxic Müller cells, secreted angiogenic factors, and neighboring endothelial cells in the regulation of MMP-2 in retinal NV, and identify MMP-2 as a target for the treatment of PDR.
Introduction

Diabetic retinopathy (DR) is the most common microvascular complication in the diabetic population. Development of DR is directly related to the duration of diabetes; by 20 years nearly all diabetic patients have DR (1). Current approaches to prevent and/or treat DR include optimizing control of blood glucose and screening of high-risk patients for early identification of retinopathy (2). Despite these efforts, DR is the leading cause of blindness among working-age adults in the developed world (3).

DR is classified as non-proliferative (NPDR) or proliferative (PDR). Sustained hyperglycemia is the major initiator for the development of NPDR (4). However, PDR is an ischemic retinopathy and develops when the oxygen demand of the inner retina exceeds oxygen supply, and results in the upregulation of angiogenic factors that promote neovascularization (NV) (5). PDR manifests clinically with retinal NV (figure S1A) that, if left untreated, can result in profound vision loss.

Retinal NV in PDR occurs at the junction between perfused and non-perfused (ischemic) retina (figure S1B), and results in the growth of fibrovascular tissue on the surface of the retina (figure S1C). Standard of care for patients with PDR is panretinal laser photocoagulation (PRP), a destructive procedure in which peripheral (ischemic) retina is sacrificed to preserve central vision (6). PDR can progress even after PRP treatment (figure S1D), and remains the most difficult consequence of diabetic eye disease to treat, highlighting the need for new therapeutic options for patients with PDR (6).

Work from several labs has resulted in our current appreciation for the central role of the transcriptional activator, hypoxia-inducible factor (HIF), in pathological angiogenesis (7). HIF regulates the expression of hypoxia-inducible genes that promote NV. The remarkable success of
therapies targeting the HIF-regulated gene product vascular endothelial growth factor (VEGF) for the treatment of diabetic macular edema (DME) highlights the importance of HIF in diabetic eye disease (8). While anti-VEGF therapy can delay and even prevent the progression to PDR (9), it has not yet been shown to be sufficient alone to treat PDR (10). Recent evidence further suggests that chronic inhibition of VEGF activity may have unwanted long-term consequences for the vulnerable neurosensory retina (11), prompting exploration for additional HIF-dependent therapeutic targets for PDR patients.

In this regard, the extensive interplay among endothelial cells (ECs), secreted factors, and the extracellular matrix (ECM) is an emerging target for anti-angiogenic therapies. In particular, ECM proteolysis has been implicated as one of the first and most sustained activities involved in pathological angiogenesis (12). Proteolysis by matrix metalloproteinases (MMPs), zinc-dependent endopeptidases that degrade various components of the ECM, has been reported to play an important role in PDR (13). However, despite well-designed studies aimed at improving our understanding of the role of these proteases in eye disease, contradictory results from several investigations have made it difficult to determine the relative contribution of MMPs to ocular NV. Consequently, reasonable disagreement remains as to whether – and which – MMPs are valid therapeutic targets for the treatment of PDR. Here we investigated the relationship between hypoxia, HIF, and MMP regulation in the ischemia-driven retinal NV observed in patients with PDR, with the goal of identifying targets for the treatment of this vision-threatened disease.
Research Design and Methods

Constructs and Reagents

Recombinant mouse (rm) and human (rh)VEGF and MMP-2 and VEGF ELISA kits were obtained from R&D Systems. Predesigned control (scrambled) and VEGF siRNA sequences were obtained from Qiagen. Digoxin and desferioxamine (DFO) were obtained from Sigma Aldrich. 1,4-dihydrophenonthrolin-4-one-3-carboxylic acid (1,4-DPCA) and dimethyloxalylglycine (DMOG) were obtained from Cayman Pharmaceuticals.

Cell Culture

MIO-M1 cells were a generous gift from Astrid Limb (University College London Institute of Ophthalmology). Isolation of primary Müller cells was performed as previously described (14). Immortalized human umbilical vein ECs (iHUVEC) were obtained from Lonza and cultured according to the manufacturer’s protocols.

Mice

8-week-old, pathogen-free female C57BL/6 mice (The Jackson Laboratory) and timed pregnant C57BL/6 mice (E14) (Charles River Laboratories) were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Johns Hopkins University Animal Care and Use Committee. Oxygen-induced retinopathy (OIR) experiments were performed as previously described (15). A subset of mice was given daily intraperitoneal (i.p.) injections of vehicle or 2 mg/kg of digoxin, a dose previously demonstrated to decrease HIF expression in the eye (16). Intraocular injections with 1 µl of rmVEGF (200 ng/µl) were performed on 8-week old
female C57BL/6 mice with a nanofil syringe (World Precision Instruments) using a 36G beveled needle under sterile conditions.

**Western Blot and Zymography**

Cell and neurosensory retina lysates were subjected to 4%-15% gradient SDS-PAGE (Invitrogen). Immunoblot assays were performed with primary antibodies specifically recognizing HIF-1α and MT1-MMP (Abcam), MMP-2 (Millipore) and GAPDH (Fitzgerald). MMP-2 enzymatic (gelatinase) activity in conditioned media was measured by zymographic assay as previously described (17), using Novex Zymogram Gel (Invitrogen).

**Quantitative Real-time RT-PCR**

mRNA was isolated from cultured cells or isolated retinas with RNeasy Mini Kit (Qiagen) and cDNA was prepared with MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and MyiQ Real-Time PCR Detection System (Bio Rad).

**Immunohistochemistry and Immunofluorescence**

Immunohistochemical detection of HIF-1α (Abcam), MMP-2 (Santa Cruz), CD31 (BD Pharmingen), and CD34 (Covance) was performed in paraffin-embedded human tissue and cryopreserved mouse tissue sections using ABC system (Dako) as previously described (18). Immunofluorescence detection of CD31, GFAP, (Sigma), HIF-1α, VEGF (Santa Cruz) and MMP-2, was performed on retina flat mounts or cryopreserved mouse tissue sections as previously described (19, 20). Immunofluorescence was performed using goat anti-mouse Alexa
F 555, goat anti-rabbit Alexa F 488, and goat anti-rat Alexa F 647 (Invitrogen) in combination with DAPI (Invitrogen). Images were captured using the confocal microscope meta 710 LSM (Carl Zeiss Inc).

Patient Samples

Institutional Review Board approval from the Johns Hopkins University School of Medicine was obtained for all patient samples and images used in this study. Aqueous and serum samples were collected from consenting patients undergoing cataract or vitrectomy surgery. Serum samples were allowed to clot for 30 minutes prior to centrifugation (1200 x g for 10 minutes).

ELISAs

ELISAs for MMP-2 and VEGF were performed according to the manufacturer’s protocols. Aqueous humor and serum were diluted 1:10 for the MMP-2 ELISA. Aqueous humor was diluted 1:10 for the VEGF ELISA. Undiluted serum was used for the VEGF ELISA.

Statistical Analysis

In all cases, results are shown as mean ± SD from at least three independent experiments. Western blot scans are representative of at least three independent experiments. Statistical analysis was performed with Prism 4.2 software (GraphPad). 

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Results

Hypoxia and HIF induce MMP-2 expression in the OIR mouse model

The OIR mouse model has previously been shown to reproduce the retinal ischemia that characterizes PDR (15). Briefly, vascularization of the posterior retina is impaired during the hyperoxia phase (P7-P12), leading to the ischemic phase (P13-P15), in which the oxygen demand of the inner retina exceeds oxygen supply (figure S2A). The subsequent angiogenic response results in retinal NV at the junction of perfused and non-perfused retina that is remarkably similar to that observed in patients with PDR (figure S2B). In a recent screen for inhibitors of the transcriptional enhancer HIF-1, the cardiac glycoside digoxin was observed to potently inhibit HIF-1α protein synthesis and, in turn, the expression of HIF-regulated genes (21). Daily treatment of mice with i.p. injections of digoxin at doses previously demonstrated to decrease HIF expression in the eye (16) results in a decrease in retinal NV (figure S2C; (16)). The OIR model therefore provides a model to examine the role of hypoxia, HIF, and HIF-regulated gene products in the pathogenesis of retinal NV in PDR.

ECM proteolysis has been implicated as one of the first and most sustained activities required for pathological angiogenesis (12). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play an important role in ECM degradation, an essential step for new vessels to escape from retina and invade the vitreous cavity, which is a hallmark of PDR. Mmp-2 – but not Mmp-9 – mRNA levels were markedly increased in the neurosensory retina during the ischemic phase (P13-P15) compared to levels immediately following hyperoxia exposure (P12; figure 1A). MMP-2 protein levels also peaked during the ischemic phase (figure 1B). Immunohistochemical analysis of P14 OIR eyes demonstrated an increase in MMP-2 in the posterior (ischemic) but not the peripheral (perfused) inner retina (figure 1C). Localized MMP-2 expression persisted
adjacent to areas of NV following the ischemic phase (P17; figure 1C and S3A).

To assess whether ischemic upregulation of MMP-2 in the inner retina is due to stabilization of the oxygen-sensitive HIF-1α subunit, beginning at P12 we treated OIR mice with daily i.p. injections of digoxin, which resulted in decreased HIF-1α protein accumulation in the posterior inner retina compared to untreated animals within 24 hours of initiating treatment (figure 1D and S3B). Mmp-2 mRNA expression was potently inhibited by digoxin treatment compared to untreated OIR mice (figure 1E), suggesting that induction of Mmp-2 mRNA expression requires HIF.

**Hypoxia stabilizes HIF-1α but does not affect MMP-2 expression in Müller cells**

We next set out to identify the cell(s) that express MMP-2 in the ischemic inner retina. Although the inner retina is composed of several cell types, increased HIF-1α protein was observed in the posterior ischemic inner retina in areas in which there was also an increase in the expression of the intermediate filament protein, glial fibrillary acidic protein (GFAP; figure 2A). GFAP is expressed in astrocytes, but also in injured or “activated” Müller glial cells. We therefore hypothesized that hypoxic Müller cells in the mouse inner retina were responsible for HIF-1α protein stabilization and Mmp-2 mRNA and protein expression in the OIR model. To directly assess whether HIF-1α regulates expression of MMP-2 in injured (hypoxic) retinal Müller cells, we isolated primary murine Müller cell cultures from the neurosensory retinas of P0-P5 C57BL/6 mice and exposed these cells to relative hypoxia (3% O₂), which led to increased HIF-1α protein levels and nuclear localization (figure 2B). Nonetheless, Mmp-2 mRNA levels did not increase following exposure of the primary murine Müller cells to hypoxia (figure 2C). These results were confirmed using MIO-M1 cells, a previously characterized immortalized
human Müller line (figure 2D and E). These results suggested that hypoxic Müller cells may not be responsible for Mmp-2 mRNA and protein expression in ischemic retinal disease.

**MMP-2 expression is induced by hypoxia in ECs**

Inspection of the inner retina in OIR eyes demonstrated that MMP-2 expression co-localized with the EC marker CD31 (figure 3A). We therefore examined whether hypoxia induces MMP-2 expression in immortalized human umbilical vein ECs (iHUVECs). Exposure of iHUVECs to hypoxia resulted in increased HIF-1α protein accumulation and nuclear localization (figure 3B). MMP-2 mRNA levels were also increased following exposure of iHUVECs to hypoxia (figure 3C). This induction was markedly decreased in iHUVECs exposed to hypoxia that were pre-treated with digoxin (figure 3D), suggesting that hypoxic induction of MMP-2 mRNA expression in ECs requires HIF.

In addition to HIF-1α, a number of transcription factors (e.g., NFκB, CREB, AP-1, p53, SP-1, and SP-3) are also activated either directly or indirectly by hypoxia. To confirm that hypoxic stabilization of HIF-1α was responsible for the induction of MMP-2 expression in ECs, we induced endogenous HIF stabilization in iHUVECs by inhibiting prolyl hydroxylases (negative regulators of HIF-1α) with 1,4-DPCA, DMOG, or DFO (figure 3E). Pharmacological stabilization of endogenous HIF-1α resulted in induction of MMP-2 mRNA expression in iHUVECs cultured under non-hypoxic (20% O2) conditions (figure 3E). Collectively, these observations demonstrate that HIF stabilization is necessary and sufficient for the promotion of MMP-2 expression in hypoxic ECs.

**MMP-2 expression by ECs is induced by secreted factors from Müller cells**
While hypoxia stabilizes HIF-1α in both hypoxic Müller glial and ECs, these results suggest that upregulation of MMP-2 expression by HIF-1 occurs primarily in ECs. However, retina vasculogenesis is a complex process that involves interplay among glial and ECs (22, 23). Indeed, careful examination of the retinal vasculature of adult mice reveals an intimate relationship between glial cells and retinal blood vessels (figure 4A). A similar relationship is also seen between retinal glial cells and neovascular buds in OIR mice (figure 4B). We therefore explored whether MMP-2 expression may be further enhanced through cooperation between Müller glia and ECs. To address this question, we co-cultured MIO-M1 cells with iHUVECs under non-hypoxic or hypoxic conditions, and measured the levels of MMP-2 mRNA expression in these co-cultures. Co-culture of MIO-M1 cells with iHUVECs was sufficient to induce a modest increase in MMP-2 mRNA expression levels under normoxic (20% O₂) conditions (figure 4C). Exposing these cells to hypoxia (1% O₂) further increased MMP-2 mRNA expression levels.

To test the hypothesis that increased MMP-2 mRNA expression in ECs was increased in response to factors released by hypoxic Müller cells, we exposed iHUVECs to conditioned media from MIO-M1 cells that were cultured at 20% or 1% O₂. iHUVECs treated with conditioned media from hypoxic MIO-M1 cells demonstrated significantly increased MMP-2 mRNA levels compared to iHUVECs treated with conditioned media from noxmoxic MIO-M1 cells (figure 4D). This result suggested that a secreted factor produced by hypoxic Müller cells promotes increased MMP-2 expression in ECs.

**VEGF is necessary and sufficient for Müller cells to promote MMP-2 expression by ECs**

We next set out to identify the factor(s) secreted by hypoxic Müller cells that induce
MMP-2 expression in neighboring ECs. It has been previously suggested that VEGF may play an important role in regulating expression of MMPs during tumor angiogenesis (24). VEGF expression was detected in the inner retina adjacent to retinal vessels in the OIR model (figure 5A). Primary murine and immortalized human Müller cells exposed to hypoxia had increased Vegf mRNA and protein levels compared to control cells (figure 5B and C).

We therefore examined whether inhibition of VEGF secretion by hypoxic Müller cells with RNA interference (RNAi) would affect the ability of media conditioned by these cells to promote MMP-2 expression in ECs. We observed that inhibition of VEGF mRNA expression in hypoxia-treated MIO-M1 cells with RNAi potently blocked the ability of conditioned media from these cells to induce MMP-2 mRNA expression in treated iHUVECs (figure 5D). Moreover, treatment of iHUVECs with rhVEGF resulted in an increase in MMP-2 mRNA expression and MMP-2 protein secretion (figure 5E and F), but did not affect MMP-9 mRNA expression (figure 5G).

VEGF induces MMP-2 enzymatic activity in ECs

Induction of MMP-2 mRNA results in expression of the inactive pro-MMP-2. Affecting MMP-2 enzymatic activity further requires the participation of membrane-type 1 (MT1)-MMP (also known as MMP-14), which is encoded by a known HIF target gene (25). MT1-MMP initiates the activation pathway by converting pro-MMP-2 into an activation intermediate that undergoes autocatalytic conversion to generate the mature (active) MMP-2 (25). Conversely, endogenous tissue inhibitors of metalloproteinases (TIMPs) inhibit MMP-2 activity. Specifically, active TIMP-2 binds to MMP-2 in a 1:1 stoichiometric ratio (26). Normally, there is a tight balance between MMP-2 and TIMP-2, but in pathological conditions this balance can be altered,
resulting in an excess of activated MMP-2. Exposure of iHUVECs to hypoxia induced both \textit{MT1-MMP} and \textit{TIMP-2} mRNA expression (figure 6A). Regulation of \textit{Mtl-mmp} and \textit{Timp-2} mRNA in the OIR model during the ischemic phase was less robust, but was also suggestive of an increase in \textit{Timp-2} mRNA (figure S4). This suggested that hypoxic induction of MMP-2 (and MT1-MMP) expression may be compensated by a balanced induction of TIMP-2 expression.

Of note, postnatal development of the normal mouse retinal vasculature is also driven by hypoxia, yet NV is not observed; rather, there is an orderly and predictable progression of retinal angiogenesis. Interestingly, \textit{Vegf} (and \textit{Mmp-2}) mRNA levels increased only modestly (less than 5- and 3-fold, respectively) during the ischemic phase (P1-P5) of postnatal retinal development (figure S5). This increase was balanced by a similar increase in \textit{Timp-2} mRNA levels. In contrast, during the ischemic phase of the OIR model, there is a more marked increase in \textit{Vegf} (almost 30-fold) and \textit{Mmp-2} (5- to 10-fold; see figure 1) mRNA expression. This suggests that a marked (and rapid) increase of VEGF may promote MMP-2 expression and activity, which then leads to the development of retinal NV.

To further examine the regulation of MMP-2 activity by VEGF \textit{in vitro}, we treated iHUVECs with rhVEGF and observed an increase in \textit{MT1-MMP} mRNA levels (figure 6B) and protein expression (figure 6C). However, unlike exposure to hypoxia, treatment of iHUVECs with rhVEGF did not affect \textit{TIMP-2} mRNA levels, suggesting that VEGF induction of MMP-2 (and MT1-MMP) expression is not compensated by an increase in TIMP-2 expression. Similarly, treatment of iHUVECs with conditioned media from hypoxic Müller cells also resulted in an increase in \textit{MT1-MMP} mRNA levels but did not affect \textit{TIMP-2} mRNA levels, whereas inhibition of VEGF secretion by hypoxic Müller cells with RNAi blocked the ability of conditioned media to induce \textit{MT1-MMP} mRNA expression (figure 6D). Zymography assays on the conditioned media...
media from iHUVECs treated with rhVEGF demonstrated an increase in MMP-2 gelatinase activity (figure 6E). Similar results were observed in primary human retinal ECs (figure S6). Collectively, these results suggest that in pathological NV, VEGF induces MMP-2 enzymatic activity by inducing its expression, while also upregulating the expression of the MMP-2 activating protein MT1-MMP, but not affecting expression of the MMP-2 inhibitor TIMP-2.

**VEGF is sufficient to induce MMP-2 expression in retinal NV in vivo**

In NV, MMPs enable the advancing front of retinal endothelial tip cells to invade through the basement membrane and ECM (12). To examine if in the absence of hypoxia VEGF alone was sufficient to promote MMP-2 expression and function by retinal endothelial tip cells in vivo, we injected rmVEGF into the vitreous of adult mice (figure 7A). *Mmp-2* mRNA levels were increased in the neurosensory retina of mice following intravitreal injection with rmVEGF compared to control (PBS)-treated mice (figure 7A). Expression of MMP-2 was detected in the inner retina of VEGF-treated eyes (Figure 7B) and localized to the tip cells of new vessels (figure 7C).

**MMP-2 expression is elevated in patients with PDR**

MMP-2 expression was detected in 4/4 autopsy eyes from patients with a known diagnosis of PDR (figure 8A), but was not detected in adjacent normal retinal vessels in these patients (figure 8A, inset). Motivated by these results, we next examined the levels of VEGF and MMP-2 in the eyes of patients with known PDR compared to diabetic patients without DR or to normal controls (Table S1). The levels of cytokines measured in the aqueous humor of patients with diabetic eye disease has previously been reported to reflect the levels measured in the
vitreous of these patients (27). We observed increased VEGF and MMP-2 levels in the aqueous of diabetic patients with PDR compared to control patients (p<0.05; figure 8B and C). Moreover, MMP-2 levels were significantly elevated in PDR patients whether or not they had prior treatment with PRP (p<0.01; figure 8D). Neither VEGF nor MMP-2 levels were increased in the serum of diabetic patients (with or without PDR) compared to control patients (figure 8E).
Discussion

Proteolytic activity of the ECM by both MMPs and non-MMPs facilitates degradation of the basement membrane, matrix remodelling, and cell migration and invasion, all of which are essential for pathological angiogenesis (28). Work on MMPs from several labs has helped broaden our appreciation for the contribution of this group of proteases to pathological angiogenesis in the eye. MMPs appear to play a dual role in the development of DR. In the earlier stages (prior to the development of NV), MMPs may have an intracellular role in which they facilitate apoptosis of retinal capillary cells, resulting in pericyte and EC loss, characteristic of early DR (29-33). However, in later stages, MMPs likely function as extracellular proteases facilitating the development of NV in PDR (13).

Much of our appreciation for the role of the MMPs in retinal NV has come from work examining knockout mice; however, results from these studies have proven difficult to interpret. Retinal NV in the OIR model was reported to be reduced in Mmp-2 null mice by two groups (34, 35) but unaffected by a third group (36). Studies examining the role of MMP-9 in retinal NV have also been conflicting: NV in the OIR model was reported to be reduced in Mmp-9 knockout mice by one group (34) but unaffected by a second group (35). These contradictory results may be due to differences in the strains of mice and to compensatory upregulation of other genes. Recent work also suggests that MMP-9 is required for the release from the bone marrow of endothelial progenitor cells (EPCs), which are essential for the development of NV (37). Thus, MMP-9 may not play a direct role in retinal NV, but rather may be required for the release of the EPCs that populate NV lesions. Ultimately, studies of retinal NV using knockout mice have not provided a clear picture as to the role of MMPs in retinal NV.

Studies demonstrating the expression of specific MMPs in PDR tissue are similarly not
sufficient to conclude that these MMPs are active in retinal NV. The promotion of ECM proteolysis by MMPs is very tightly regulated; it is therefore equally important to determine whether their inhibitors or activators – and their enzymatic activity – are also modulated. It is also essential that key events that trigger the expression of these genes be also delineated. Understanding the role of MMPs in retinal NV is further complicated by the participation of multiple cell types that may contribute different components of the proteolysis machinery. The consequence of these obstacles is that reasonable disagreement remains as to whether – and which – MMPs are valid therapeutics targets for the treatment of PDR.

Here we provide evidence demonstrating that the transcriptional activator, HIF-1, can induce MMP-2 expression in ischemic retinopathies in two ways: 1) stabilization of HIF-1α in hypoxic ECs leads to increased expression of MMP-2 in these cells; and 2) accumulation of HIF-1α in hypoxic Müller cells leads to increased VEGF secretion which, in turn, upregulates MMP-2 expression in neighboring ECs (figure S7). This may further help explain why Mmp-2 mRNA levels peak later than Vegf mRNA levels in the OIR model. We further demonstrate that while hypoxic induction of MMP-2 expression in ECs is balanced by the simultaneous upregulation of TIMP-2, a negative regulator of MMP-2, VEGF induction of MMP-2 is not balanced by an increase in TIMP-2. Accordingly, MMP-2 enzymatic activity is markedly increased by treatment of ECs with VEGF, which was sufficient to promote MMP-2 expression in endothelial tip cells in vivo. Of note, TIMP-2 has also been shown to play a role in promoting the activation of MMP-2 by MT1-MMP (38, 39) Additionally, MT1-MMP itself is a collagenase and could play a direct (MMP-2-independent) role the development of fibrovascular proliferation in PDR (40). Additional studies will be necessary to further characterize the complex regulation of the function of these proteases in diabetic eye disease.

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Elevated expression of MMPs was previously reported within retinal neovascular tissue in patients with PDR, as well as in the aqueous of patients with PDR (41-48). We demonstrate here that MMP-2 levels are increased in PDR patients compared to controls, regardless of whether or not PDR patients had prior treatment with PRP, suggesting that MMP-2 is a therapeutic target even in patients who have had prior treatment for PDR. Serum levels of MMP-2 in diabetic patients (with or without PDR) were almost identical to control patients, indicating that the increased levels of MMP-2 in the eyes of PDR patients were an ocular (not systemic) consequence of diabetes.

Collectively, our findings provide molecular and cellular mechanisms underlying the regulation of MMP-2 in retinal NV and suggest that MMP-2 is a valid target for the treatment of PDR. In support of the latter, prior work has demonstrated that broad-spectrum pharmacological inhibition of MMPs prevents retinal NV in animal models (34, 49, 50). In light of our results, we propose that therapies specifically targeting MMP-2 could become an important and effective part of the complex approach to prevent or treat NV in patients with PDR.
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S.M. and A.S. designed experiments; M.R., X.X., K.J., S.B., F.K., T.M., I.B., and S.H. performed experiments; Y.D., D.B., S.S, and A.S. provided clinical samples; M.R., X.X., K.J., T.M., S.M., and A.S. interpreted data. G.L., G.S., and S.M reviewed and edited the manuscript. A.S. wrote the manuscript.

There are no conflicts of interest to report.

Akrit Sodhi, M.D., Ph.D., is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Figure Legends

Figure 1: HIF-dependent MMP-2 expression during ischemic phase of OIR model. (A) RT-PCR of *Mmp-2* and *Mmp-9* mRNA from the neurosensory retina of OIR animals at P12 to P15 normalized to *cyclophilin B* mRNA, and reported as fold induction compared to P12. (B) Representative western blot of MMP-2 protein accumulation from the neurosensory retina of OIR animals at P12 to P15. Paired lanes represents lysates from two eyes from two separate animals. GAPDH was used as a loading control. (C) Representative immunohistochemical analysis of MMP-2 (black arrows; blue chromogenic substrate) in the retina of OIR eyes during the ischemic phase at P14 (posterior retina, *left*, and peripheral retina, *center*) and during the neovascular phase, P17 (*right*). (D) Representative immunohistochemical analysis of HIF-1α (brown chromogenic substrate) in the retina of P13 OIR eyes (*center*) compared to P13 control eyes (*left*). HIF-1α expression was inhibited with daily intraperitoneal injections of digoxin (*right*). (E) RT-PCR of *Mmp-2*, *Mmp-9*, and *Vegf* mRNA from the neurosensory retina of OIR animals at P12 to P14 with (+dig) or without daily intraperitoneal injection of digoxin, normalized to *cyclophilin B* mRNA, and reported as fold induction compared to P12. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05; ** p < 0.01.

Figure 2: Stabilization of HIF-1α in hypoxic Müller cells does not lead to MMP-2 expression. (A) Representative immunofluorescent analysis of HIF-1α in the retina of P13 OIR eyes compared to control P13 eyes. (B) Western blot and immunofluorescence for HIF-1α in primary murine Müller cells exposed to hypoxia. GAPDH was used as a loading control. (C) *Mmp-2* mRNA levels from primary murine Müller cells exposed to hypoxia, normalized to
cyclophilin B mRNA, and reported as fold induction compared to cells exposed to 20% O₂ (C).

(D and E) Similar studies were performed using immortalized human Müller (MIO-M1) cells. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05.

Figure 3: Stabilization of HIF-1α in hypoxic vascular ECs leads to increased MMP-2 mRNA levels. (A) Representative immunofluorescent analysis of MMP-2 in the retina of P13 and P14 OIR eyes compared to control P13 eyes. Retinal vasculature was labeled with anti-CD31, an EC marker. (B) Western blot and immunofluorescence for HIF-1α in iHUVECs exposed to hypoxia. GAPDH was used as a loading control. (C) VEGF and MMP-2 mRNA normalized to β-actin mRNA from iHUVECs exposed to hypoxia, reported as fold induction compared to control cells exposed to 20% O₂. (D) Left, western blot for HIF-1α (above) and VEGF mRNA normalized to β-actin mRNA and reported as fold induction compared to control cells exposed to 20% O₂ (below) from iHUVECs exposed to hypoxia, pre-treated with digoxin. GAPDH was used as a loading control for the western blot. Right, MMP-2 mRNA normalized to β-actin mRNA from iHUVECs exposed to hypoxia, pre-treated with digoxin, and reported as fold induction compared to control cells. (E) Left, western blot for HIF-1α (above) and VEGF mRNA normalized to β-actin mRNA and reported as fold induction compared to control cells (below) in iHUVECs treated with 1,4-DPCA and DMOG, or DFO for 8 hours. GAPDH was used as a loading control for the western blot. Right, MMP-2 mRNA normalized to β-actin mRNA from iHUVECs treated with 1,4-DPCA, DMOG, or DFO, for 8 hours, and reported as fold induction compared to control cells. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05; ** p <
0.01.

**Figure 4: Cooperation between retinal Müller glial cells and ECs increases MMP-2 mRNA levels in co-cultured cells.** (A and B) Representative immunofluorescent staining for the EC marker CD31 and the glial cell marker GFAP in the retina of adult (A) and OIR (B) mice. (C) MMP-2 mRNA levels by RT-PCR in co-cultures of MIO-M1 cells with iHUVECs under normoxic or hypoxic conditions. (D) iHUVECs were cultured at 20% O$_2$ in the presence of conditioned media (for 4 or 8 hours) from MIO-M1 cells exposed to 20% O$_2$ or 1% O$_2$ (for 12 to 48 hours, as indicated). Levels of MMP-2 mRNA (normalized to β-actin mRNA) are reported as fold induction compared to untreated controls. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05; ** p < 0.01.

**Figure 5: VEGF is necessary and sufficient for Muller cells to induce MMP-2 mRNA expression in ECs.** (A) Representative immunofluorescent analysis of VEGF in the retina of P13 OIR eyes compared to control P13 eyes. The EC marker CD31 highlights retinal vasculature. (B) Vegf mRNA levels by RT-PCR in primary murine Müller cells exposed to hypoxia (normalized to cyclophilin B mRNA) are reported as fold induction compared to untreated controls. (C) VEGF mRNA and protein secretion in immortalized human Müller (MIO-M1) cells exposed to hypoxia reported as fold induction compared to untreated controls. (D) VEGF and MMP-2 mRNA levels in iHUVECs treated with conditioned media from MIO-M1 cells exposed to hypoxia (0 to 6 hours) with or without RNAi against VEGF mRNA reported as fold induction compared to untreated controls. (E and F) MMP-2 mRNA (E) and secreted protein (F) levels
following treatment of iHUVECs with increasing doses of rhVEGF (for 6 hours) or increasing duration of treatment (with 10 ng rhVEGF) reported as fold induction compared to untreated controls. (G) MMP-9 mRNA levels following treatment of iHUVECs with increasing doses of rhVEGF (for 6 hours) or increasing duration of treatment (with 10 ng rhVEGF) reported as fold induction compared to untreated controls. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05; ** p < 0.01.

Figure 6: VEGF promote increased MMP-2 activity by increasing MT1-MMP expression in ECs. (A) MT1-MMP and TIMP-2 mRNA levels, normalized to β-actin mRNA following exposure of iHUVECs to hypoxia for 0 to 8 hours, reported as fold induction compared to untreated controls. (B) MT1-MMP and TIMP-2 mRNA levels, normalized to β-actin mRNA following treatment of iHUVECs with increasing doses of rhVEGF for 6 hours or increasing duration of treatment with 10 ng rhVEGF, reported as fold induction compared to untreated controls. (C) Western blot for MT1-MMP in iHUVECs treated with increasing doses of rhVEGF (for 8 hours) or increasing duration of treatment (with 10 ng rhVEGF). GAPDH was used as a loading control. (D) MT1-MMP and TIMP-2 mRNA levels (RT-PCR) in iHUVECs treated with conditioned media from MIO-M1 cells exposed to hypoxia (0 to 6 hours) with or without RNAi against VEGF mRNA reported as fold induction compared to untreated controls. (E) MMP-2 enzymatic (gelatinase) activity following treatment of iHUVECs with increasing doses of rhVEGF (for 6 hours) or increasing duration of treatment (with 10 ng rhVEGF), reported as fold induction compared to untreated controls. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test:
* p < 0.05; ** p < 0.01.

**Figure 7:** VEGF promotes increased MMP-2 expression in retinal NV *in vivo* and in human PDR tissue. *(A)* Above, schematic demonstrating intravitreal injections of rmVEGF or PBS control. *Below,* Mmp-2 mRNA from the neurosensory retina of animals injected with rmVEGF over time normalized to cyclophilin B mRNA, and reported as fold induction compared to control (PBS-injected) eyes. *(B and C)* Representative immunofluorescent analysis of MMP-2 (green arrows) in cross section *(B)* and flat mount *(C)* from eyes injected with rmVEGF or PBS (control). The EC marker CD31 highlights tip cells of retinal microvasculature (arrow heads). All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05.

**Figure 8:** Increased MMP-2 expression in patients with PDR. *(A)* Representative images from immunohistochemical analysis of MMP-2 expression in NV (black arrows) but not in normal retinal vessels (blue arrow) in the retina of 4/4 eyes with NV and a known diagnosis of PDR (see inset). No primary antibody was used for the negative control. *(B)* VEGF levels in the aqueous humor of non-diabetic patients (Control), diabetic patients without DR (Diabetic), and diabetic patients with PDR (PDR). *(C)* MMP-2 levels in the aqueous humor of these patients. *(D)* MMP-2 levels in the aqueous humor of non-diabetic patients (Control) compared to diabetic patients with PDR (PDR) who were not previously treated with PRP (Untreated) or those who had previous PRP treatment (Treated). *(E)* Left, VEGF levels in the serum of non-diabetic patients (Control) and diabetic patients with PDR (PDR). Right, MMP-2 levels in the serum of non-diabetic patients (Control), diabetic patients without DR (Diabetic) and diabetic patients

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with PDR (PDR). All experiments were performed in duplicate. Student t-test: * p < 0.05. Multiple t-tests: * p < 0.05; ** p < 0.01.
Supplementary Materials

Cell Culture

Primary human retinal ECs were obtained from Angio-Proteomie and cultured according to the manufacturer’s protocols.

Immunohistochemistry and Immunofluorescence

Immunofluorescence detection of pimonidazole (HypoxyProbe; HPI) was performed on cryopreserved mouse tissue sections as previously described (19, 20).
Supplementary Figure Legends

Figure S1: Proliferative diabetic retinopathy, retinal non-perfusion and retinal neovascularization. (A and B) Fundus photo (A) and fluorescein angiographic image (B) from a patient with untreated PDR with extensive NV of the disc (NVD; blue arrows) and elsewhere (NVE; red arrows). High magnification of fluorescein angiographic image demonstrates areas of non-perfusion (asterisks) adjacent to NVE (red arrows). (C) Representative H and E image of fibrovascular membrane on the surface of the retina in a patient with PDR. (D) Left, fundus photos of a patient with PDR with NVD (blue arrow) and NVE (red arrows) and pre-retinal hemorrhage (H) prior to PRP treatment. Right, following PRP treatment (black arrows), fibrous membranes with residual traction (T) on the retina remain.

Figure S2: HIF-dependent promotion of retinal neovascularization in induced retinopathy (OIR) model. (A) Representative cross section images from P12 and P14 OIR eyes. (red = CD31; green = HypoxyProbe). (B) Representative cross-section images of P17 OIR eyes (black arrows = neovascular buds). (C and D) Reduced neovascular vessels in P17 OIR (C) or control (D) eyes by daily intraperitoneal injection of digoxin. n = 3 animals in each group.

Figure S3: High magnification images of immunohistochemical analysis of MMP-2 and HIF-1α expression in OIR model. (A and B) High magnification of representative immunohistochemical analysis of MMP-2 (A) or HIF-1α (B) in the retina of OIR eyes at P14. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group.
Figure S4: Regulation of *Mt1-mmp* and *Timp-2* mRNA during ischemic phase of OIR model. (A and B) RT-PCR of *Mt1-mmp* (A) and *Timp-2* (B) mRNA from the neurosensory retina of OIR animals at P12 to P14 normalized to *cyclophilin B* mRNA, and reported as fold induction compared to P12. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05.

Figure S5: Modest induction of *Vegf*, *Mmp-2*, and *Timp-2* (but not *Mt1-mmp*) mRNA during vascular development in the mouse eye. (A) RT-PCR of *Mmp-2*, *Mmp-9*, *Timp-2*, *Mt1-Mmp*, and *Vegf* mRNA from the neurosensory retina of developing mouse eye, normalized to *cyclophilin B* mRNA, and reported as fold induction compared to P1. (B) Representative immunofluorescence analysis of MMP-2 in the retina of the developing mouse eye. All experiments were performed in duplicate are representative of at least 3 independent experiments. n = 3 animals in each group. Student t-test: * p < 0.05.

Figure S6: VEGF promotes increased MMP-2 activity by increasing MT1-MMP expression in primary human retinal ECs. (A) Western blot for HIF-1α and MMP-2 in primary human retinal ECs exposed to hypoxia for 0 to 8 hours. GAPDH was used as a loading control. (B and C) *VEGF* (B) and *MMP-2* (C) mRNA normalized to *β-actin* mRNA from primary human retinal ECs exposed to hypoxia, reported as fold induction compared to control cells exposed to 20% O$_2$. (D - G) MMP-2 mRNA levels (D and E) and protein secretion (F and G) following treatment of primary human retinal ECs with increasing doses of rhVEGF (for 6 hours; D and F) or increasing duration of treatment (with 10 ng rhVEGF; E and G) reported as fold induction compared to untreated controls. (H) Western blot for MT1-MMP in primary human
retinal ECs treated with increasing doses of rhVEGF (for 8 hours). GAPDH was used as a loading control. (I) MMP-2 enzymatic (gelatinase) activity following treatment of primary human retinal ECs with increasing doses of rhVEGF (for 6 hours) reported as fold induction compared to untreated controls. All experiments were performed in duplicate and are representative of at least 3 independent experiments. Student t-test: * p < 0.05; ** p < 0.01.

**Figure S7: Regulation of MMP-2 activity by hypoxia.** HIF-1α is stabilized and localizes to the nucleus in hypoxic Müller cells where it induces expression and secretion of VEGF (1). VEGF acts on neighboring ECs to promote NV and edema (2). HIF-1α is stabilized and localizes to the nucleus in hypoxic ECs where it directly induces Pro-MMP-2 and MT1-MMP expression (3A). This is balanced by hypoxic induction of the MMP-2 inhibitor, TIMP-2. Pro-MMP-2 and MT1-MMP (but not TIMP-2) expression are also induced by VEGF (3B). MT1-MMP cleaves inactive Pro-MMP-2 to release the active *MMP-2 which can then promote NV.
Figure S2

A

OIR P12

OIR P14

B

posterior

posterior

posterior

posterior

C

P17 OIR

P17 OIR + digoxin

D

P17 control

GFAP

Hypoxy Probe

CD31

GFAP

DAPI

periphery

periphery

periphery
Figure 1

A

MMP-2 RNA (fold induction)

|    | P12 | P13 | P14 | P15 |
|----|-----|-----|-----|-----|
| 0  | 5   | 10  | 15  | 15  |

MMP-9 RNA (fold induction)

|    | P12 | P13 | P14 | P15 |
|----|-----|-----|-----|-----|
| 0  | 5   | 10  | 15  | 15  |

B

MMP-2

GAPDH

C

P14 posterior

P14 periphery

P17 (neovascularization)

D

P13 control posterior

P13 OIR peripheral

P13 OIR + digoxin posterior

E

MMP-2 RNA (fold induction)

|    | p12 | p13 | p13+dig | p14 | p14+dig |
|----|-----|-----|---------|-----|---------|
| 0  | 5   | 10  | 15      | 15  | 15      |

MMP-9 RNA (fold induction)

|    | p12 | p13 | p13+dig | p14 | p14+dig |
|----|-----|-----|---------|-----|---------|
| 0  | 5   | 10  | 15      | 15  | 15      |

VEGF RNA (fold induction)

|    | p12 | p13 | p13+dig | p14 | p14+dig |
|----|-----|-----|---------|-----|---------|
| 0  | 5   | 10  | 15      | 15  | 15      |
Figure S3

A

MMP-2  P14 posterior

P14 periphery

P17 (neovascularization)

B

HIF-1α  P13 OIR

Peripheral (pr)

Transition (t)

Posterior (ps)
Figure 2

A. control

B. Hypoxia (hours)

C. MMP-2 RNA (fold induction)

D. HIF-1α

E. MMP-2 RNA (fold induction)
Figure 3

A) DAPI, MMP-2, CD31, and merge images showing control, P13, and P14 conditions.

B) Western blot analysis showing HIF-1α and GAPDH levels under normoxia and hypoxia conditions.

C) Bar graph showing fold induction of VEGF and MMP-2 RNA under hypoxia conditions.

D) Western blot analysis showing HIF-1α and GAPDH levels under hypoxia conditions with and without Digoxin treatment.

E) Western blot analysis showing HIF-1α and GAPDH levels under hypoxia conditions with 1,4-DPCA, DMOG, DFO, and Digoxin treatments.
Figure 4

**Panel A**: Immunofluorescence images showing CD31 and GFAP staining in the retinal vessels of P17 OIR mice. The images are merged to demonstrate the co-localization of CD31 and GFAP in the neovascular buds.

**Panel B**: Similar immunofluorescence images for P17 OIR Neovascular buds showing the expression of CD31 and GFAP.

**Panel C**: Graph showing the fold induction of MMP-2 RNA in iHUVECs exposed to hypoxia in the presence or absence of media conditioned by MIO-M1 cells. The graph indicates significant induction of MMP-2 RNA in hypoxic conditions.

**Panel D**: Bar graph depicting the fold induction of MMP-2 RNA in iHUVECs exposed to hypoxia for varying durations of time, with or without media conditioned by MIO-M1 cells. The graph shows a dose-dependent induction of MMP-2 RNA.

Exposure of MIO-M1 cells to hypoxia (hrs): 0, 12, 24, 48
Duration of treatment of iHUVECs with media conditioned by MIO-M1 cells (hrs): 4, 8, 12, 24, 48

*Significance levels are denoted by asterisks and error bars represent standard error of the mean.
Figure 5

A. P13 Control vs. P13 OIR

B. VEGF RNA (fold induction)

C. VEGF Secretion (fold induction)

D. VEGF secretion (fold induction)

E. MMP-2 RNA (fold induction)

F. MMP-2 Secretion (fold induction)

G. MMP-9 RNA (fold induction)
Figure 6

A. MT1-MMP RNA (fold induction) in response to Hypoxia (hours).

B. MT1-MMP RNA (fold induction) in response to VEGF (ng).

C. VEGF (ng) and MT1-MMP GAPDH expression.

D. MT1-MMP RNA (fold induction) with Hypoxia (hours).

E. Gelatinase activity and VEGF (ng) and hours.

* indicates statistical significance.
Figure S4

A

MT1-MMP mRNA (fold induction)

P12  P13  P14

B

TIMP-2 mRNA (fold induction)

P12  P13  P14

*
Figure S5

A

**MMP-2 RNA (fold induction)**

**MMP-9 RNA (fold induction)**

**TIMP-2 RNA (fold induction)**

**MT1-MMP RNA (fold induction)**

**VEGF RNA (fold induction)**

B

P1  P3  P5  P7  P21

**CD31**

**GFAP**

**MMP-2**

**merge**
Figure S6

A

Hypoxia (hours)

HIF-1α

MMP-2

GAPDH

B

Hypoxia (hours)

VEGF mRNA (fold induction)

C

Hypoxia (hours)

MMP-2 mRNA (fold induction)

D

VEGF (ng)

MMP-2 mRNA (fold induction)

E

VEGF (hours)

MMP-2 mRNA (fold induction)

F

VEGF (ng)

MMP-2 secretion (fold induction)

G

VEGF (hours)

MMP-2 secretion (fold induction)

H

MT1-MMP

GAPDH

I

VEGF (ng)

Gelatinase activity

Diabetes
Figure 7

A

Intravitreal Injection

rmVEGF or PBS

MMP-2 RNA (fold induction)

0 24 48

hours post injection

* * *

B

Control

VEGF

CD31

MMP-2

merge

C

Control

VEGF

CD31

MMP-2

merge
### Table S1: Characteristics of Subjects

|                | Controls (n=20) | DM, No DR (n=10) | DM, PDR (n=17) |
|----------------|----------------|-----------------|----------------|
| **Average age (range)** | 64.8 (46-87)   | 66.4 (54-80)    | 51.3 (31-71)   |
| **Gender M:F**  | 5:14           | 4:6             | 9:5            |
| **cCVD**        | 5              | 5               | 6              |

*a* includes one patient who had an aqueous sample taken from the same eye twice

*b* includes one patients who had an aqueous sample taken from the same eye twice and one patient who had an aqueous sample taken from the same eye twice and from the other eye once

*c* includes any patient with a history of hypertension, hypercholesterolemia, coronary artery disease, or cerebral vascular accident
Figure 8

A negative CD34

MMP-2

Concentration (pg/mL)

VEGF Serum

Concentration (pg/mL)

MMP-2 Serum

Concentration (ng/mL)

MMP-2 Aqueous Humor Concentration (ng/mL)

VEGF Aqueous Humor Concentration (pg/mL)

MMP-2 Aqueous Humor Concentration (ng/mL)

Control

Diabetic

PDR

Control

Diabetic

PDR

Control

Non-PRP Treated PDR

PRP Treated PDR

Control

Untreated PDR

Treated PDR

Control

PDR

Control

Diabetic

PDR
Figure S7

Proteolysis
Neovascularization
HIF-1α
VEGF
Müller cell

Hypoxia

Survival
Proliferation
Migration
Neovascularization
Endothelial cell

Pro-MMP-2
MT1-MMP
*MMP-2
TIMP-2

3B

Diabetes
Figure S3

(A) Graphs showing fold induction of MMP-2, MMP-9, TIMP-2, MT1-MMP, and VEGF RNA levels in samples P1, P3, P5, P7, and P21.

(B) Images showing immunofluorescent staining for DAPI (nuclei), CD31 (endothelial cells), GFAP (astrocytes), MMP-2, and merged images for Diabetes samples P1, P3, P5, P7, and P21.
|                      | Controls  | DM, No DR | DM, PDR  |
|----------------------|-----------|-----------|----------|
|                      | (n=20)    | (n=10)    | (n=17)   |
| Average age (range)  | 64.8 (46-87) | 66.4 (54-80) | 51.3 (31-71) |
| Gender M:F           | 5:14      | 4:6       | 9:5      |
| CVD                  | 5         | 5         | 6        |

*a* includes one patient who had an aqueous sample taken from the same eye twice.

*b* includes one patient who had an aqueous sample taken from the same eye twice and one patient who had an aqueous sample taken from the same eye twice and from the other eye once.

*c* includes any patient with a history of hypertension, hypercholesterolemia, coronary artery disease, or cerebral vascular accident.