Role of Matrix Metalloproteinase-9 in the Development of Diabetic Retinopathy and Its Regulation by H-Ras

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PURPOSE. Diabetes activates a small molecular weight G-protein, H-Ras, in the retina and its capillary cells, and H-Ras activation is implicated in the apoptosis of retinal capillary cells. Matrix metalloproteinase (MMP)-9 is regulated by H-Ras, and in diabetes its activation is associated with increased vascular permeability. The goal of this study was to investigate the role of sustained activation of MMP-9 in the pathogenesis of diabetic retinopathy and to illustrate the mechanism through which it is upregulated in diabetes.

METHODS. Retinal MMP-9 activation and its tissue inhibitor, TIMP-1, were quantified in streptozotocin-induced diabetic rats. Inhibition of H-Ras by simvastatin on diabetes-induced activation of H-Ras was evaluated. The mechanism by which diabetes regulates retinal MMP-9 was confirmed by determining the effect of genetic or pharmacologic regulation of H-Ras on its activation in retinal endothelial cells.

RESULTS. In rats, MMP-9 was activated and expression of TIMP-1 was decreased in the retina and its microvasculature at both 2 months and 12 months of diabetes. In retinal endothelial cells, high glucose activated MMP-9, and inhibition of its activation (by pharmacologic inhibitor or siRNA) ameliorated accelerated apoptosis. Inhibition of H-Ras, both in diabetic rats (simvastatin) and in isolated endothelial cells (H-Ras siRNA), abrogated the activation of MMP-9 and prevented the reduction of TIMP-1.

CONCLUSIONS. Hyperglycemia-induced activation of MMP-9 accelerates apoptosis of retinal capillary cells, a phenomenon that predicts the development of diabetic retinopathy, and the activation of MMP-9 is downstream of H-Ras. Characterizing the role of MMP-9 in the development of diabetic retinopathy will help explore novel molecular targets for future pharmacological interventions. (*Invest Ophthalmol Vis Sci.* 2010;51:4320–4326) DOI:10.1167/iov.09-4851

Diabetic retinopathy is a multifactorial disease, and hyperglycemia is considered the major factor in its development. Retinal microvascular (pericytes and endothelial cells) and other noncapillary cells (e.g., Müller cells and glial cells) are lost selectively through apoptosis before histopathology characteristic of diabetic retinopathy is observed.^{1–3} Although many abnormalities, including increased mitochondrial dam-

age and activation of other pathways, are implicated in the loss of retinal capillary cells in diabetes,^{4–8} the exact mechanism remains illusive.

H-Ras, a GTP-binding protein of small molecular weight, is known to act as a biological switch for cellular processes.⁹ Its actions are mediated through the activation of multiple downstream effector signaling cascades, which in turn regulate transcription factors and gene expression.¹⁰ Transient activation of H-Ras is considered to result in cell differentiation, whereas sustained activation in cell growth and its activation in selective compartments is considered to have distinct physiological outcomes.¹¹ Our previous studies have shown that H-Ras is activated in the retina and its microvasculature in animal models of diabetic retinopathy and that activation of H-Ras contributes to the accelerated apoptosis of retinal capillary cells in diabetes, suggesting a major role of this G-protein in the development of diabetic retinopathy.^{12–14} Activation of H-Ras requires its membrane anchoring by isoprenylation, which involves the activity of 3-hydroxy 3-methylglutaryl (HMG)-CoA reductase.¹⁵ We have shown that retinal H-Ras activation in diabetes occurs through its translocation to the membrane, which can be inhibited by an (HMG)-CoA reductase inhibitor, simvastatin.¹⁶

H-Ras is shown to upregulate MMP-9, the largest and most complex member of the MMP family, which regulates a variety of cellular functions, including proliferation, differentiation, and angiogenesis.¹⁷ MMP-9 is induced or repressed by a variety of soluble factors such as cytokines and growth factors.¹⁸ Endogenous tissue inhibitors of MMPs (TIMPs) regulate their activation, and TIMP-1 shows greater preference for MMP-9 than any other MMP.¹⁹ The levels of MMP-9 are significantly elevated in the plasma and retinas of patients with diabetes²⁰ and in the vitreous and retinas of patients with diabetic retinopathy.^{21–23} Increased MMP-9 is also observed in the retina of a mouse model of neovascularization and in the human retina showing active neovascularization.²⁴ Our recent studies have shown that the activation of retinal MMP-2, another member of the MMP family, in diabetes is under the control of superoxide.²⁵ However, the exact mechanism by which MMP-9 contributes to the development of diabetic retinopathy is unclear.

The purpose of this study was to investigate the role of MMP-9 in the pathogenesis of diabetic retinopathy by investigating the activation of retinal MMP-9 at 2 months of diabetes (duration at which alterations in the blood-retinal barrier are present²²) and at 12 months of diabetes (retinal capillary cell apoptosis and pathology are observed^{1–3,7}) in rats. The mechanism by which diabetes upregulates retinal MMP-9 is investigated by determining the effect of regulation of H-Ras (by molecular and pharmacologic means) on its activation in retinal capillary cells exposed to high glucose. In addition, simvastatin is shown to inhibit H-Ras activation in the retina.^{16,26} The role of H-Ras in regulating MMP-9 activation in diabetes is also confirmed by analyzing the retinas of diabetic rats receiving diets supplemented with simvastatin.

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METHODS

Rats

Wistar rats, made diabetic with streptozotocin, were used to obtain retinas and microvessels. Diabetic rats received powder diet (5001; Ralston Purina, St. Louis, MO) supplemented with 10 mg/kg/d simvastatin (Merck Research Laboratories, West Point, PA) or without any supplementation. These diets were initiated soon after the establishment of diabetes (3–4 days after the administration of streptozotocin). A group of diabetic rats receiving simvastatin or no simvastatin, and their age-matched normal rats, were killed 2 months after initiation of the experiment by overdose of pentobarbital, and each retina was removed immediately. The retina from one eye was frozen in liquid nitrogen for biochemical measurements, and the retina from the other eye was used immediately to prepare microvessels by hypotonic method. For long-term study, diabetic rats and their age-matched normal rats were killed 12 months after the induction of diabetes. Retinal capillary cell apoptosis and pathology are observed at this duration of diabetes in rats.^{3,7,25} Each group had 10 to 12 rats, and the entire colony of rats (normal, diabetic, and diabetic with simvastatin diet) received fresh powder diet weekly. Glycated hemoglobin (GHb) was measured at approximately 2 months after the induction of diabetes and every 3 months thereafter using affinity columns (kit 442-B; Sigma Chemical, St. Louis, MO). Treatment of the animals conformed to the National Institute of Health Principles of Laboratory Animal Care, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and institutional guidelines.

Retinal Microvessels

Freshly isolated retina was incubated in distilled water for 1 hour at 37°C, followed by 5 minutes' incubation with DNase (2 mg/mL). Because of limited availability of tissue, microvessels were prepared only from the diabetic rats and their age-matched normal controls at 2 months' duration. The retinal vasculature was isolated under a microscope by repetitive inspiration and ejection through Pasteur pipettes. Retinal blood vessels isolated by this hypotonic method show a normal complement of nuclei and are devoid of nonvascular materials.²⁵

Isolated Retinal Endothelial Cells

Bovine retinal endothelial cells were grown in Dulbecco's modified Eagle medium containing 15% fetal bovine serum (heat inactivated), 5% Nu-serum, 50 µg/mL heparin, 50 µg/mL endothelial cell growth supplement, and 1% antibiotic/antimycotic. Confluent cells from third to sixth passages were incubated in 5 mM glucose (normal) or 20 mM glucose (high) for 4 days in the presence or absence of either farnesylation inhibitor (25 µM FTI-277 or 10 µM manumycin) or MMP-9 inhibitor (4 nM MMP-9 inhibitor-I [MMP-I; EMD-Calbiochem, San Diego, CA]). Osmotic control included cells incubated in 20 mM mannitol instead of 20 mM glucose. Media were changed every 48 hours, and each experiment was repeated with three to four different cell preparations.

Transfection of Endothelial Cells

Endothelial cells from the third to the fifth passages were transfected with small interfering RNA of either H-Ras (H-Ras-siRNA) or MMP-9 (MMP-9-siRNA) or with either wild-type or dominant-negative (N17) mutant of H-Ras. Transfection reagents and siRNA duplex were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The transfection complexes were prepared by adding 20 to 80 pmol H-Ras siRNA or MMP-9-siRNA diluted in siRNA transfection medium and 2 to 8 µL siRNA transfection reagent incubated for 30 minutes at room temperature. The cells were washed with the serum and antibiotic-free transfection medium, and the transfection complex was overlaid onto the cells. The cells were incubated with the complex for 7 to 8 hours at 37°C. To assess the specificity of the RNA interference method, parallel incubations were carried out using nontargeting

control siRNA (scrambled siRNA) and the transfection reagent alone (mock). After transfection the media were replaced with 5 mM and 20 mM glucose media, and the cells were incubated for 4 additional days. At the end of the incubation period, the cells were rinsed with phosphate-buffered saline and harvested. These methods are routinely used in our laboratory.^{13,16}

MMP-Gelatinase Assay

MMP-9 gelatinase activity was measured in the culture medium or tissue lysate by zymography. Equal amounts of protein (15–25 µg) were electrophoresed under nonreducing conditions into 10% SDS-PAGE gels polymerized with 1 mg/mL gelatin. The gels were washed with 2.5% Triton X-100 for 30 minutes with gentle shaking, followed by a 30-minute wash with distilled water. The gels were then incubated overnight at 37°C in substrate buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂, and 0.02% NaN₃ and were stained with Coomassie blue (Simply Blue Safe Stain; Invitrogen, Carlsbad, CA). This was followed by destaining with distilled water.²⁵ Active MMP-9 (92 kDa) band was detected in the zymogram and quantified using digitizing software (Un-Scan-It Gel; Silk Scientific, Orem, UT).

Gene Expression

Gene expressions of MMP-9 and TIMP-1 were quantified by real-time PCR using the assays for the bovine species (*TaqMan*; Applied Biosystems Inc., Foster City, CA). In a total volume of 20 µL, 90 ng cDNA was mixed with the specific assay and universal PCR mix (Master; Applied Biosystems Inc.) and was amplified in 96-well plates using a sequence detection system (ABI-7500; Applied Biosystems Inc.) under standard PCR conditions, as described earlier. Data collection was performed during the last 30 seconds. Each sample was analyzed in triplicate. 18S rRNA was validated as an appropriate housekeeping gene. Threshold lines were automatically adjusted to intersect amplification lines in the linear portion of the amplification curves, and the software was automatically recorded the cycle to threshold (C_t). The fold change in gene expression relative to normal was calculated using the ddC_t method. These methods have been routinely used in our laboratory.^{14,25}

Activation of H-Ras by Raf-1 Binding

The relative abundance of active, GTP-bound Ras was quantified using a Raf-1 binding assay kit (Cytoskeleton, Denver, CO). Equal amounts of retinal homogenate protein (80–120 µg) were added to Raf-1RBD, and the Raf-RBD/GTP-Ras complex was pulled down by glutathione affinity beads. The beads were resuspended in Laemmli reducing sample buffer and boiled for 5 minutes. The amount of activated H-Ras was determined by Western blot using H-Ras-Pan specific antibody.^{13,14,16}

Apoptosis

Cell death was determined with a commercial kit (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics, Indianapolis, IN). Monoclonal antibodies directed against DNA and histones, respectively, were used to quantify the relative amounts of mononucleosomes and oligonucleosomes generated from apoptotic cells. The cytoplasmic fraction of the cells was transferred onto a streptavidin-coated microtiter plate and incubated for 2 hours at room temperature with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled anti-histone. The plate was washed and incubated (ABTS; Roche Diagnostics), and absorbance was measured at 405 nm.^{12,25}

Caspase-3 Activity

Activity of caspase-3 was quantified by measuring the formation of *p*-nitroanilide by the cleavage of Ac-DEVD-pNA (Biomol Research Laboratories, Plymouth Meeting, PA) at 405 nm. Each sample was analyzed in duplicate.

Statistical Analysis

Results are presented as mean $\pm$ SD and analyzed statistically using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U* test for multiple group comparisons. Similar conclusions were achieved by using ANOVA with the Fisher exact test or the Tukey test.

RESULTS

Activation of MMP-9 in the Retina and Its Microvessels

The gelatinase activity of MMP-9 is increased by approximately 50% in the retina obtained from rats diabetic for 2 months (Fig. 1a). Similar activation was observed when the duration of diabetes was extended to 12 months (a duration when histopathology characteristic of diabetic retinopathy can be observed in this animal model). In the same retina samples, the gene expression of MMP-9 was elevated by 50% to 60% (Fig. 1a), and that of its tissue inhibitor (TIMP-1) was decreased by approximately 35% (Fig. 1b) compared with the values obtained from age-matched normal control rats. Similar diabetes-induced increases in MMP-9 gelatinase activity and gene transcripts were observed in the retinal microvessels, the site of histopathology associated with diabetic retinopathy (Fig. 1c).

Effect of Simvastatin on Diabetes-Induced Activation of Retinal MMP-9

In addition to inhibiting the activation of retinal H-Ras in diabetes, the HMG-CoA reductase inhibitor simvastatin, which inhibits membrane anchoring of Ras through a farnesylation-associated mechanism, also inhibited the gelatinase activity of MMP-9 (Fig. 2). Simvastatin administration to diabetic rats, however, had no effect on the severity of hyperglycemia; glycated hemoglobin values were 11% to 12% in diabetic rats with or without simvastatin compared with 5% to 6% in normal control rats.

Effect of High Glucose on MMP-9 and Its Regulators in Retinal Endothelial Cells

Exposure of bovine retinal endothelial cells to high glucose levels increased MMP-9 gelatinase activity by 60% to 65% compared with the activity observed in the cells exposed to normal glucose under identical conditions (Fig. 3), and this was accompanied by a concomitant reduction in the gene expression of its tissue inhibitor TIMP-1 (Fig. 4). Incubation of retinal endothelial cells with 20 mM mannitol, however, did not have any effect on the activation of MMP-9, confirming that the activation of MMP-9 by high glucose was not caused by increased osmolarity (Fig. 3).

Inclusion of a cell-permeable and selective inhibitor of MMP-9, MMP-I, during high glucose exposure of retinal endothelial cells attenuated increases in the gelatinase activity of MMP-9 (Fig. 3). Similar results were obtained from the retinal cells that were transfected with MMP-9-siRNA before they were exposed to high glucose. In contrast, however, transfection with the scrambled siRNA did not prevent glucose-induced activation of MMP-9, and the values obtained were significantly higher than those obtained from the cells expressing MMP-9-siRNA (Fig. 3). Exposure of cells transfected with MMP-9-siRNA or scrambled siRNA to normal glucose, however, did not affect MMP-9 activity, and the values obtained were similar to those obtained from nontransfected cells exposed to normal glucose.

Effect of MMP-9 Activation on Apoptosis of Endothelial Cells Experienced in High-Glucose Conditions

Exposure of retinal endothelial cells to high glucose increased their apoptosis by more than 75% (Fig. 5a) and activated the

FIGURE 1. MMP-9 is upregulated in the retina in diabetes. (a) MMP-9 gelatinase activity and gene expression (by real-time PCR) were measured in retinas obtained from rats that had diabetes for 2 months (Diab-2) or 12 months (Diab-12). Histogram represents gene expression of MMP-9 adjusted to that of 18sRNA. (b) TIMP-1 gene expression was measured in the same retina samples by real-time PCR using assays for the bovine species. (c) Gelatinase activity and gene expression of MMP-9 were measured in the retinal microvessels prepared from rats that had been diabetic for 2 months. Values obtained from normal rat retina/microvessels are considered as 100%. Each measurement was made in duplicate, and the values are represented as mean $\pm$ SD obtained from six or more rats in each group. **P* < 0.05 compared with normal.

apoptosis execution enzyme caspase-3 by 50% (Fig. 5b). This glucose-induced apoptosis of retinal capillary cells was significantly attenuated when the high glucose exposure period was supplemented with MMP-I. This inhibition effect of MMP-9 on the glucose-induced apoptosis of retinal endothelial cells was confirmed by transfection experiments in which the apoptosis of capillary cells was abrogated by the transfection of endothe-

FIGURE 2. Simvastatin inhibits diabetes-induced activation of retinal H-Ras and MMP-9. Retinas harvested from diabetic rats receiving diet supplemented with or without simvastatin were used to measure the activation of H-Ras by Raf-1 binding assay and that of MMP-9 by measuring its gelatinase activity. Each sample was measured in duplicate using retinas from five to seven rats in each group, and the graph represents mean $\pm$ SD. * $P < 0.05$ compared with normal. # $P < 0.05$ compared with diabetes.

lial cells with MMP-9-siRNA but not with the scrambled siRNA (Fig. 5a). These results strongly suggest that, in high glucose conditions, MMP-9 has a proapoptotic role in retinal endothelial cells.

Effect of H-Ras on Glucose-Induced MMP-9 Activation

Transfection of retinal endothelial cells with H-Ras-siRNA prevented glucose-induced activation of MMP-9, but transfection

FIGURE 3. MMP-9 is activated in retinal endothelial cells in high-glucose conditions. Bovine retina endothelial cells from the third to the sixth passages were incubated in 5 mM glucose or 20 mM glucose medium for 4 days in the presence or absence of 4 nM MMP-I. For siRNA experiments, the cells were transfected with MMP-9-siRNA (MMP-siR) or scrambled siRNA (Scramb), followed by incubation in 5 mM or 20 mM glucose for 4 days. Cells treated with the transfection reagents alone are identified as Mock. At the end of the incubation, the medium was collected to quantify the gelatinase activity of MMP-9. Each measurement was made in duplicate in at least three different cell preparations. Values obtained from the untransfected cells incubated in 5 mM glucose are considered as 100% (control). 5G, 5 mM glucose; 20 G, 20 mM glucose; 20 mannitol, 20 mM mannitol. * $P < 0.05$ compared with the values obtained from the cells incubated in 5 mM glucose.

FIGURE 4. High glucose attenuates TIMP-1 in retinal endothelial cells. Gene expression of TIMP-1 was quantified in the retinal endothelial cells incubated in 5 mM or 20 mM glucose for 4 days in the presence or absence of 25 μ M FTI-277 or 10 μ M manumycin (by real-time PCR using assays for bovine species). The level of TIMP-1 gene expression was adjusted to that of 18sRNA in each sample. Each measurement was performed in three to four different cell preparations. Values obtained from the cells incubated in 5 mM glucose are considered as 100% (control). * $P < 0.05$ compared with the values obtained from the cells incubated in 5 mM glucose.

with scrambled RNA had no effect on MMP-9 activity. In addition, though overexpression of the wild-type mutant of H-Ras exerted minimal effects on the glucose-induced activation of MMP-9, such deleterious effects of high glucose were abolished in cells transfected with the dominant-negative (N17) mutant of H-Ras (Fig. 6).

To further confirm the role of H-Ras, inhibition of H-Ras activation by either of its functional inhibitors, FTI-277 and manumycin, in addition to preventing the activation of H-Ras, ameliorated glucose-induced activation of MMP-9 (Fig. 6). In the same cell preparations, both FTI-277 and manumycin prevented glucose-induced reduction in TIMP-1 (Fig. 4). Taken together, these data suggest that glucose-induced activation of MMP-9 is under the control of H-Ras.

DISCUSSION

Matrix metalloproteinases, a class of approximately 25 zinc-dependent proteinases, degrade at least one component of the extracellular matrix and are considered to regulate a variety of cellular functions, including morphogenesis, wound healing, angiogenesis, and metastasis.^{17,27,28} Here we suggest a novel function and a regulation mechanism for one of the most complex—and the largest—member in the pathogenesis of diabetic retinopathy, the major cause of blindness in young adults. Our results show that in diabetes, MMP-9 is activated and its tissue inhibitor, TIMP-1, is decreased in the retina and its microvasculature—the site of diabetic retinopathy-associated abnormality²⁹—when retinopathy is observed in diabetic rats.³ MMP-9 activation increases the apoptosis (which appears to occur through caspase-3 activation) of retinal capillary cells, which can be ameliorated by its pharmacologic inhibitor and siRNA. Further, we provide the possible mechanism of MMP-9 activation in diabetes; our results clearly demonstrate that MMP-9 activation in the retina and its endothelial cells is under the control of H-Ras. The inhibition of H-Ras in both in vivo and in vitro models of diabetic retinopathy abrogates the activation of MMP-9 and prevents any reduction in TIMP-1.

FIGURE 5. Inhibition of glucose-induced activation of MMP-9 prevents apoptosis of retinal endothelial cells. (a) Apoptosis was measured with cell death ELISA by measuring cytoplasmic histone-associated DNA fragments using a commercial assay kit. (b) Caspase-3 activity was determined in the cells by measuring the cleavage of the substrate Ac-DEVD-pNA. Each experiment was repeated with at least three different cell preparations and measurements performed in duplicate. Values obtained from the cells incubated in 5 mM glucose are considered as 100% (control). * $P < 0.05$ and # $P < 0.05$ compared with the values obtained from the cells incubated in 5 mM glucose (5 G) and 20 mM glucose (20 G), respectively.

Diabetes has been shown to activate MMPs in various tissues, including heart, kidney and plasma.^{20,30,31} Increased MMP-9 activity is reported in the retinas and vitreous of patients with diabetic retinopathy^{21,24} and in the retinas of diabetic rodents early during the course of the disease, before histopathologic evidence of retinopathy.²² In addition, MMP-9 is upregulated in retinal microvascular cells cultured under high-glucose conditions.^{22,32} Here we show that MMP-9 remains active at 12 months of diabetes in rats, a duration when capillary cell apoptosis and histopathologic findings characteristic of diabetic retinopathy can be observed in rats, suggesting that MMP-9 activation has a major role in the development of diabetic retinopathy. How increased MMP-9 activates apoptosis remains to be explored, and the possibility that MMP-9 is activated by the disruption of mitochondrial connexin-43 protein and the degradation of mitochondrial membrane potential³³ cannot be ruled out because the downregulation of connexin-43 is considered an early trigger for inducing apoptosis in retinal capillary cells.³⁴

MMP-9 is associated with inflammatory cell migration and extracellular matrix degradation, and the pro-form of MMP-9 is

significantly elevated in the neovascular retinal membranes.³⁵ Increased MMP-9 is also observed in retinas with active neovascularization.²⁴ Activation of retinal MMP is postulated to facilitate the increase in vascular permeability through proteolytic degradation of the tight junction protein-occludin and disruption of the overall tight junction complex,^{22,32,36} and this is considered an early event in the development of diabetic retinopathy. Further, in the pathogenesis of diabetic retinopathy, endothelial cell invasion occurs during neovascularization, and MMP-9 is associated with inflammatory cell migration and extracellular matrix degradation.³⁷ Here we show that MMP-9 activation has another important role in the pathogenesis of diabetic retinopathy. The inhibition of MMP-9 activation prevents the accelerated apoptosis of retinal capillary cells, a phenomenon that precedes the development of diabetic retinopathy.³ This proapoptotic role of MMP-9 in hyperglycemic conditions occurs through the activation of caspase-3 and is consistent with our recent studies demonstrating a similar role of MMP-2, another member of the gelatinase subfamily of MMP.²⁵ Thus, MMP-9 activation has the potential to contribute to the development of diabetic retinopathy by altering both the

FIGURE 6. Regulation of H-Ras activation prevents MMP-9 activation. Retinal endothelial cells from the third to the sixth passages were incubated in 5 mM glucose or 20 mM glucose medium for 4 days in the presence or absence of 25 μ M FTI-277 or 10 μ M manumycin. For siRNA experiments, the cells were transfected with H-Ras-siRNA (Ras-siR) or scrambled siRNA (Scramb) or H-Ras mutant that is dominant negative (N17) or wild-type followed by incubation in 5 mM or 20 mM glucose for 4 days. Controls were also run using the cells incubated with the transfection reagents alone before exposure to 5 mM or 20 mM glucose for 4 days (Mock). At the end of the incubation, the gelatinase activity of MMP-9 was quantified in the medium. Each measurement was made in duplicate in at least three different experiments. Values obtained from the untransfected cells incubated in 5 mM glucose are considered as 100% (control). * P < 0.05 compared with the values obtained from the cells incubated in 5 mM glucose.

early event—vascular permeability—and the late event—capillary cell apoptosis. We must acknowledge that our in vitro experiments show more capillary cells undergoing glucose-induced apoptosis,^{1,3} but this might not truly reflect the in vivo phenomenon observed in the diabetic retina. However, our studies and those of others have suggested that most mechanism-based data are comparable between the in vitro and in vivo model systems of diabetic retinopathy.^{4,6,12–14,23,25,32}

H-Ras, a small molecular weight G-protein, is activated in the retina and its capillary cells, and activation of H-Ras accelerates apoptosis of retinal capillary cells in diabetes.^{12–14} In rat liver epithelial cells, H-Ras activation is shown to regulate MMP-9 expression,^{34,38} and filamin A-induced constitutive activation of the Ras modulates the production of MMP-9.³⁹ Overexpression of the H-Ras in human fibroblasts correlates with the upregulation of MMP-9,⁴⁰ and inhibition of H-Ras function in cancer cells inhibits pro-MMP-9.⁴¹ Here we provide data showing that inhibition of H-Ras function (by pharmacologic or genetic manipulation) prevents activation of MMP-9, suggesting that in the pathogenesis of diabetic retinopathy, activation of MMP-9 is regulated by H-Ras, and MMP-9 appears to be downstream of H-Ras. The mechanism by which diabetes-induced retinal H-Ras activates MMP-9 is unclear but may include an NF- κ B activation step because MMP-9 has a functional enhancer element-binding site for NF- κ B,³⁸ and the activation of H-Ras modulates NF- κ B in retinal endothelial cells, which can be prevented by the inhibition of H-Ras function.^{12–14}

Simvastatin inhibits H-Ras downstream signaling molecules,^{26,42} and in the retina it inhibits the metabolic abnormalities associated with the development diabetic retinopathy, including leukocyte-endothelial cell interactions, blood retinal barrier breakdown, and ICAM-1 expression.^{43,44} Our previous study has shown that the administration of simvastatin to diabetic rats prevents the translocation of retinal H-Ras from cytosol to membrane fraction.¹⁶ Here we show that the administration of simvastatin, in addition to inhibiting H-Ras activation, also inhibits the activation of MMP-9, further supporting the regulatory role of H-Ras in the activation of MMP-9. This beneficial effect of simvastatin on MMP-9 is consistent with reports by others showing the protective effect of simvastatin against diabetic nephropathy through the suppression of renal MMP-9⁴⁵ and on the downregulation of MMP-9 in abdominal aortic aneurysm by statins.⁴⁶

In summary, MMP-9 acts as proapoptotic factor in the accelerated loss of retinal capillary cells seen in the pathogenesis

of diabetic retinopathy. We have identified a mechanism by which diabetes activates MMP-9; the results have demonstrated that the activation of MMP-9 is downstream of H-Ras. Understanding the mechanism responsible for the pathogenesis of diabetic retinopathy by characterizing the role of MMP-9 in retinal capillary cell death will help explore novel molecular targets for future pharmacologic interventions to slow this devastating complication of diabetes.

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References

- Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest.* 1996;97:2883–2890.
- Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes: early onset and effect of insulin. *J Clin Invest.* 1998;102:783–791.
- Kern TS, Tang J, Mizutani M, Kowluru R, Nagraj R, Lorenzi M. Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. *Invest Ophthalmol Vis Sci.* 2000;41:3972–3978.
- Kowluru RA, Abbas SN. Diabetes-induced mitochondrial dysfunction in the retina. *Invest Ophthalmol Vis Sci.* 2003;44:5327–5334.
- Behl Y, Krothapalli P, Desta T, DiPiazza A, Roy S, Graves DT. Diabetes-enhanced tumor necrosis factor- α production promotes apoptosis and the loss of retinal microvascular cells in type 1 and type 2 models of diabetic retinopathy. *Am J Pathol.* 2008;172:1411–1418.
- Takamura Y, Tomomatsu T, Kubo E, Tsuzuki S, Akagi Y. Role of the polyol pathway in high glucose-induced apoptosis of retinal pericytes and proliferation of endothelial cells. *Invest Ophthalmol Vis Sci.* 2008;49:3216–3223.
- Kowluru RA, Chan PS. Metabolic memory in diabetes—from in vitro oddity to in vivo problem: role of apoptosis. *Brain Res Bull.* 2010;81:297–302.
- Joussen AM, Doehmen S, Le ML, et al. TNF- α mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. *Mol Vis.* 2009;15:1418–1425.
- Esteban LM, Vicario-Abejon C, Fernandez-Salguero P, et al. Targeted genomic disruption of H-ras and N-ras, individually or in

- combination, reveals the dispensability of both loci for mouse growth and development. *Mol Cell Biol*. 2001;21:1444-1452.
10. Schubert S, Bollag G, Shannon K. Deregulated Ras signaling in developmental disorders: new tricks for an old dog. *Curr Opin Genet Dev*. 2007;17:15-22.
 11. Klein C, Creach K, Irintcheva V, et al. Zinc induces ERK-dependent cell death through a specific Ras isoform. *Apoptosis*. 2006;11:1933-1944.
 12. Kowluru RA, Kowluru A, Chakrabarti S, Khan Z. Potential contributory role of H-Ras, a small G-protein, in the development of retinopathy in diabetic rats. *Diabetes*. 2004;53:775-783.
 13. Kowluru RA, Kowluru A, Kanwar M. Small molecular weight G-protein, H-Ras, and retinal endothelial cell apoptosis in diabetes. *Mol Cell Biochem*. 2007;296:69-76.
 14. Kanwar M, Kowluru RA. Diabetes regulates small molecular weight G-protein, H-Ras, in the microvasculature of the retina: implication in the development of retinopathy. *Microvasc Res*. 2008;76:189-193.
 15. Alvarado Y, Giles FJ. Ras as a therapeutic target in hematologic malignancies. *Expert Opin Emerg Drugs*. 2007;12:271-284.
 16. Kowluru RA, Kanwar M. Translocation of H-Ras and its implications in the development of diabetic retinopathy. *Biochem Biophys Res Commun*. 2009;387:461-466.
 17. Dufour A, Sampson NS, Zucker S, Cao J. Role of the hemopexin domain of matrix metalloproteinases in cell migration. *J Cell Physiol*. 2008;217:643-651.
 18. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol*. 2002;37:375-536.
 19. Brew K, Dinakarandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta*. 2000;1477:267-283.
 20. Yildirim N, Sahin A, Erol N, Kara S, Uslu S, Topbas S. The relationship between plasma MMP-9 and TIMP-2 levels and intraocular pressure elevation in diabetic patients after intravitreal triamcinolone injection. *J Glaucoma*. 2008;17:253-256.
 21. Jin M, Kashiwagi K, Iizuka Y, Tanaka Y, Imai M, Tsukahara S. Matrix metalloproteinases in human diabetic and nondiabetic vitreous. *Retina*. 2001;21:28-33.
 22. Giebel SJ, Menicucci G, McGuire PG, Das A. Matrix metalloproteinases in early diabetic retinopathy and their role in alteration of the blood-retinal barrier. *Lab Invest*. 2005;85:567-607.
 23. Yang R, Liu H, Williams I, Chaqour B. Matrix metalloproteinase-2 expression and apoptogenic activity in retinal pericytes: implications in diabetic retinopathy. *Ann N Y Acad Sci*. 2007;1103:196-201.
 24. Das A, McLamore A, Song W, McGuire PG. Retinal neovascularization is suppressed with a matrix metalloproteinase inhibitor. *Arch Ophthalmol*. 1999;117:498-503.
 25. Kowluru RA, Kanwar M. Oxidative stress and the development of diabetic retinopathy: contributory role of matrix metalloproteinase-2. *Free Rad Biol Med*. 2009;46:1677-1685.
 26. Dai Y, Khanna P, Chen S, Pei XY, Dent P, Grant S. Statins synergistically potentiate 7-hydroxystaurosporine (UCN-01) lethality in human leukemia and myeloma cells by disrupting Ras farnesylation and activation. *Blood*. 2007;109:4415-4423.
 27. Nagase H, Woessner JF. Matrix metalloproteinases. *J Biol Chem*. 1999;274:21491-21494.
 28. Malesud CJ. Matrix metalloproteinases (MMPs) in health and disease: an overview. *Front Biosci*. 2006;11:1696-1701.
 29. Klein R. Hyperglycemia and microvascular and macrovascular disease in diabetes. *Diabetes Care*. 1995;18:258-268.
 30. Tyagi SC, Hayden MR. Role of nitric oxide in matrix remodeling in diabetes and heart failure. *Heart Fail Rev*. 2003;8:23-28.
 31. Thrall KM, Clay Bunn R, Fowlkes JL. Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy. *Endocrine*. 2009;35:1-10.
 32. Navaratna D, McGuire PG, Menicucci G, Das A. Proteolytic degradation of VE-cadherin alters the blood-retinal barrier in diabetes. *Diabetes*. 2007;56:2380-2387.
 33. Moshal KS, Tipparaju SM, Vacek TP, et al. Mitochondrial matrix metalloproteinase activation decreases myocyte contractility in hyperhomocysteinemia. *Am J Physiol Heart Circ Physiol*. 2008;295:H890-H897.
 34. Lee KW, Kim MS, Kang NJ, et al. H-Ras selectively up-regulates MMP-9 and COX-2 through activation of ERK1/2 and NF- κ B: an implication for invasive phenotype in rat liver epithelial cells. *Int J Cancer*. 2006;119:1767-1775.
 35. Das A, McGuire PG, Eriqat C, et al. Human diabetic neovascular membranes contain high levels of urokinase and metalloproteinase enzymes. *Invest Ophthalmol Vis Sci*. 1999;40:809-813.
 36. Beránek M, Kolar P, Tschoplova S, Kankova K, Vasku A. Genetic variations and plasma levels of gelatinase A (matrix metalloproteinase-2) and gelatinase B (matrix metalloproteinase-9) in proliferative diabetic retinopathy. *Mol Vis*. 2008;14:1114-1121.
 37. Kolaczowska E, Grzybek W, van Rooijen N, et al. Neutrophil elastase activity compensates for a genetic lack of matrix metalloproteinase-9 (MMP-9) in leukocyte infiltration in a model of experimental peritonitis. *J Leukoc Biol*. 2009;85:374-381.
 38. Yoon SO, Shin S, Lee HL, Chun HK, Chung AS. Isoginkgetin inhibits tumor cell invasion by regulating phosphatidylinositol 3-kinase/Akt-dependent matrix metalloproteinase-9 expression. *Mol Cancer Ther*. 2006;5:2666-2675.
 39. Zhu TN, He HJ, Kole S, et al. Filamin A-mediated down-regulation of the exchange factor Ras-GRF1 correlates with decreased matrix metalloproteinase-9 expression in human melanoma cells. *J Biol Chem*. 2007;282:14816-14826.
 40. Giambernardi TA, Grant GM, Taylor GP, et al. Overview of matrix metalloproteinase expression in cultured human cells. *Matrix Biol*. 1998;16:483-496.
 41. Desrosiers RR, Cusson MH, Turcotte S, Béliveau R. Farnesyltransferase inhibitor SCH-66336 downregulates secretion of matrix proteinases and inhibits carcinoma cell migration. *Int J Cancer*. 2005;114:702-712.
 42. Kang S, Kim ES, Moon A. Simvastatin and lovastatin inhibit breast cell invasion induced by H-Ras. *Oncol Rep*. 2009;21:1317-1322.
 43. Miyahara S, Kiryu J, Yamashiro K, et al. Simvastatin inhibits leukocyte accumulation and vascular permeability in the retinas of rats with streptozotocin-induced diabetes. *Am J Pathol*. 2004;164:1697-1706.
 44. Al-Shabrawey M, Bartoli M, El-Remessy AB, et al. Role of NADPH oxidase and Stat3 in statin-mediated protection against diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2008;49:3231-3238.
 45. Yao XM, Ye S, Zai ZM, et al. Simvastatin protects diabetic rats against kidney injury through the suppression of renal matrix metalloproteinase-9 expression. *J Endocrinol Invest*. In press.
 46. Evans J, Powell JT, Schwalbe E, Loftus IM, Thompson MM. Simvastatin attenuates the activity of matrix metalloproteinase-9 in aneurysmal aortic tissue. *Eur J Vasc Endovasc Surg*. 2007;34:302-303.