Hyperglycemia-Suppressed Expression of Serpine1 Contributes to Delayed Epithelial Wound Healing in Diabetic Mouse Corneas

Haijing Sun, Xiaofan Mi, Nan Gao, Chenxi Yan, and Fu-Shin Yu

Departments of Ophthalmology and Anatomy/Cell Biology, Wayne State University School of Medicine, Detroit, Michigan, United States

Correspondence: Fu-Shin Yu, Kresge Eye Institute, Wayne State University School of Medicine, 4717 St. Antoine Boulevard, Detroit, MI 48201, USA; fyu@med.wayne.edu.

Submitted: February 3, 2015

Accepted: April 6, 2015

Citation: Sun H, Mi X, Gao N, Yan C, Yu F-S. Hyperglycemia-suppressed expression of Serpine1 contributes to delayed epithelial wound healing in diabetic mouse corneas. *Invest Ophthalmol Vis Sci.* 2015;56:3383–3392. DOI:10.1167/iovs.15-16606

PURPOSE. Patients with diabetes mellitus (DM) are at an increased risk for developing corneal complications, including delayed wound healing. The purpose of this study was to characterize the expression and the function of Serpine1 and other components of urokinase plasminogen activator (uPA)–proteolytic system in delayed epithelial wound healing in diabetic mouse corneas.

METHODS. Mice of the strain C57BL/6 were induced to develop diabetes by streptozotocin, and wound-healing assays were performed 10 weeks afterward. Gene expression and/or distribution were assessed by real-time PCR, Western blotting, and/or immunohistochemistry. The role of Serpine1 in mediating epithelial wound closure was determined by subconjunctival injections of neutralizing antibodies in either normal or recombinant protein in diabetic corneas. Enzyme assay for matrix metalloproteinase (MMP)-3 was also performed.

RESULTS. The expressions of Serpine1 (PAI-1), Plau (uPA), and Plaur (uPA receptor) were upregulated in response to wounding, and these upregulations were significantly suppressed by hyperglycemia. In healing epithelia, Plau and Serpine1 were abundantly expressed at the leading edge of the healing epithelia of normal and, to a lesser extent, diabetic corneas. Inhibition of Serpine1 delayed epithelial wound closure in normal corneas, whereas recombinant Serpine1 accelerated it in diabetic corneas. The Plau and MMP-3 mRNA levels and MMP-3 enzymatic activities were correlated to Serpine1 levels and/or the rates of epithelial wound closure.

CONCLUSIONS. Serpine1 plays a role in mediating epithelial wound healing and its impaired expression may contribute to delayed wound healing in DM corneas. Hence, modulating uPA proteolytic pathway may represent a new approach for treating diabetic keratopathy.

Keywords: cDNA array, diabetic keratopathy, Serpine1 (PAI-1), corneal epithelial wound healing, MMP-3

With a rapid increase in the prevalence of diabetes mellitus (DM), ocular complications have become a leading cause of blindness in the world.¹ The ocular complications of DM are numerous and include retinopathy, cataract, uveitis, and neuro-ophthalmic disorders.² In addition to the aforementioned complications, various types of corneal disorders also are relatively common in DM patients.³ Abnormalities of the cornea, termed diabetic keratopathy, are resistant to conventional treatment regimens.^{4,5} Unlike diabetic retinopathy or cataracts, patients with diabetic keratopathy usually do not have detectable symptoms; however, once the cornea is injured, delayed epithelial wound healing is often observed.⁶ Delayed epithelial wound closure may be associated with sight-threatening complications, such as stromal opacification, surface irregularity, and microbial keratitis.⁴ Moreover, although a growing number of diabetic patients are requesting laser surgery for elective vision correction each year, the US Food and Drug Administration considers diabetes to be a relative contraindication to the surgery; postoperative infections and/or impaired wound healing are two risk factors for the concern.⁷ Early studies revealed that the abnormalities of the cornea include alterations in the epithelial

basement membrane, such as thickening,⁸ decreased number of hemidesmosomes,⁹ and deposition of advanced glycation end products.¹⁰ For the epithelium, hyperglycemia significantly alters its structure and function, resulting in basal cell degeneration,^{8,11,12} decreased¹³ or increased¹⁴ cell proliferation, superficial punctate keratitis,¹⁵ breakdown of barrier function,¹⁶ fragility,¹⁷ recurrent erosions, and persistent epithelial defects,¹⁸ depending on the duration of DM and on the serum concentration of glycated hemoglobin HbA_{1c}. More recently, defects in growth factors, such as epidermal growth factor,¹⁹ TGF-β3,²⁰ hepatocyte growth factor,²¹ and opioid growth factor,²² in microRNAs,²³ and in proteinases such as matrix metalloproteinase (MMP)-10 and cathepsin F²⁴ were found to be associated with delayed epithelial wound healing in diabetic corneas. However, to date the mechanisms underlying diabetic keratopathy and delayed epithelial wound healing remain incompletely understood.

Hyperglycemia likely executes its adverse effects on corneal wound healing by modifying the expression of a host of wound response genes.²⁰ In a recent study, we reported the use of a genome-wide cDNA array to screen for genes, their associated pathways, and the networks affected by hyperglycemia in

epithelial cells in vivo and have identified a large group of genes differentially expressed in DM healing versus normal-healing corneal epithelial cells (CECs).²⁰ Subsequent analysis revealed that wound-induced upregulation of TGF β 3 in normal corneas was suppressed in diabetic corneas, while TGF β 1 levels remained elevated. Functional analysis indicated that TGF β 3 was required for proper wound healing in normal (NL) corneas. Supplementation with recombinant TGF β 3 accelerated epithelial wound closure in diabetic corneas via Smad and PI3K-AKT signaling pathways, autoregulation, and upregulation of *Serpine1*, a well-known TGF β target gene. Our cDNA array data revealed that Serpine1 (also termed plasminogen activator inhibitor 1 or PAI-1) is among the most highly wound-inducible (54.5-fold increase) and hyperglycemia-sensitive (3.6-fold decrease) transcripts. We propose that Serpine1 is an effector of TGF β signaling pathways that are activated in response to wounding.

Serpine1 is a serine protease inhibitor that functions as the principal inhibitor of tissue (tPA) and urokinase plasminogen activator (uPA).²⁵ The primary function of uPA/tPA is to convert plasminogen to active serine protease plasmin, a strong proteolytic enzyme that cleaves fibrin, fibronectin, thrombospondin, laminin, and von Willebrand factor. Plasmin also is known to convert the latent form of TGF- β to its active form,^{26,27} and pro-MMPs to MMPs. Serpine1 interaction with uPA/tPA blocks uPA/tPA activation, plasmin formation, and plasmin-dependent MMP activation, mostly type IV collagenases/gelatinases, MMP2, and MMP9.^{28,29} Our cDNA array study revealed that although the level of tPA (Plat) remained unchanged, uPA (Plau) and its receptor uPAR (Plaur) showed a similar pattern of expression as Serpine1. Hence, Serpine1 likely acts through the uPA proteolytic pathway to mediate epithelial wound healing in the cornea. As the major physiologic regulator of the pericellular plasmin-generating cascade,^{30,31} Serpine1 was found to modulate cellular adhesion or migration, wound healing, angiogenesis, and tumor cell metastasis.^{32,33} As such, Serpine1 has been implicated as a mediator in many processes, including fibrosis, rheumatoid arthritis, atherosclerosis, tumor angiogenesis, and bacterial infections. In the corneas, Serpine1 upregulation was reported in response to wounding³⁴ and was shown to stimulate human CEC adhesion and migration in vitro.³⁵ Serpine1 has been implicated in the pathogenesis of diabetic retinopathy^{36,37} and yet a higher Serpine1 plasma level was found to be independently associated with a lower risk of retinopathy.³⁸ Our cDNA array study also revealed that MMP-3 had a largest increase in healing corneal epithelia of euglycemia but not hyperglycemia mice. Human CECs have been shown to express MMP-3, which was implicated in the pathogenesis of human pterygium and ocular rosacea.^{39,40} Interestingly, a recent study revealed that MMP-3 was a downstream effector of Serpine1 in promoting neurovascular injury in brain trauma.⁴¹ To date, the role of Serpine1 and/or the uPA proteolytic pathway and potential involvement of MMP-3 in wound healing in normal conditions and the effects of its defects in expression during delayed wound repair and ulceration in diabetic tissues such as the cornea and the skin remain elusive.

In this report, we followed up on our genome cDNA array and TGF β 3 studies and assessed the expression and potential functions of Serpine1 using a mouse model of human type 1 diabetes. We found that, although wound-induced Serpine1 expression was greatly suppressed in diabetic corneas, addition of exogenous Serpine1 accelerated delayed wound healing, and, for the first time, regulated MMP-3 expression and enzymatic activity in wounded corneas. Our data suggested that early intervention with systemic and local therapies modulating Serpine1 and/or uPA proteolytic activities may provide hope for the better management of diabetic corneal complications.

FIGURE 1. Delayed wound healing in STZ-induced type 1 diabetic mice. B6 mice (6 weeks old) were intraperitoneally injected with 50 mg/kg STZ daily for 5 days; control mice received citrate buffer (pH 4.5). Mice were tested for the levels of blood sugar at week 4 post injection. Mice with higher than 350 mg/dL blood glucose were used for wound-healing study at week 10 post STZ injection. A 1.5-mm wound was made in the center of the cornea and the healing process was visualized with fluorescein staining of the denuded area. *Top:* Representative images are shown. *Bottom:* The wound sizes were calculated by Adobe Photoshop (V6) Magic Wand Tool and Histogram (Adobe Systems, Inc., San Jose, CA, USA) and presented as the average of the number of pixels of the fluorescent-stained area. The error bars represent the SEM ($n = 5$) and indicated P values were generated using paired Student's t -test. Five independent experiments ($n = 5$ each condition) were performed.

METHODS

Ethics Statement

All investigations using animals conformed to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health, and the guidelines of the Animal Investigation Committee of Wayne State University.

TABLE. Decreased Expression of uPA Proteolytic System in Healing CECs of Diabetic Rats

Gene	Nondiabetic		Diabetes		UW		Healing	
	UW: Healing		UW: Healing		Diabetes: Normal		Diabetes: Normal	
	P Value	Change	P Value	Change	P Value	Change	P Value	Change
<i>Plg</i>	0.9937	1.0008	0.9083	1.012	0.2898	−1.12	0.2485	−1.1325
<i>Plat</i>	0.7730	−1.0723	0.248	1.3387	0.6692	−1.1094	0.0821	−1.59265
<i>Plau</i>	0.0004	6.0305	0.0001	8.4058	0.7463	−1.1103	0.1997	−1.54762
<i>Plaur</i>	0.0002	4.4343	8.37E-5	5.1169	0.0105	−2.1023	0.0042	−2.42593
<i>Serpine1</i>	1.81E-05	54.474	0.0002	17.378	0.7827	−1.1346	0.021	−3.55668

UW, unwounded.

Animals and Induction of Diabetes

Six-week-old C57BL/6 mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA) were housed under standard conditions with continuously available water and chow (LabDiet 5001 Rodent Chow; LabDiet, Dexter, MI, USA). B6 mice were induced to develop diabetes with streptozotocin (STZ) as described.^{20,42} Mice were considered as diabetic with blood-glucose levels higher than 350 mg/dL within 4 weeks after injection and thereafter.⁴²

Corneal Epithelial Debridement Wound

Ten weeks after induction of the stable nontoxic diabetic state, DM and age-matched normal mice were anesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (70 mg/kg), along with the application of topical proparacaine to the corneas. A 1.5-mm circular wound was first demarcated with a trephine in the central cornea; the epithelium was then removed with a blunt scalpel blade under a dissecting microscope (Zeiss, Peabody, MA, USA). The blade with scraped epithelial cells was immediately immersed into liquid nitrogen and the visible “ice” was collected into an Eppendorf tube placed on dry ice with two corneas pooled in one tube, stored at -80°C . The collected cells are marked as unwounded (or 0 hour). Bacitracin ophthalmic ointment (Fougera, Melville, NY, USA) was applied to the cornea after surgery to prevent infection. The progress of wound healing was monitored by corneal fluorescence staining for epithelial defects and photographed with a slit-lamp microscope. At the end of healing, the corneas are either snap-frozen in optimum cutting temperature (OCT) compound for cryostat sectioning or marked with the same size trephine for epithelial cell collection at 22 to 24 hours post wounding (hpw). Epithelial cells within the circle were removed and collected as described for the original wounding and samples were marked as healing CECs.

RNA Extraction and Real-Time PCR

RNA was extracted from the collected epithelial cells using RNeasy Mini Kit (QIAGEN, Gaithersburg, MD, USA), according to the manufacturer's instructions. Complementary DNA was generated with an oligo(dT) primer (Invitrogen, Grand Island, NY, USA) followed by analysis using real-time PCR with the Power SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) based on expression of β -actin.

Immunohistochemistry of Mouse Corneas

Mouse eyes were enucleated and embedded in Tissue-Tek OCT compound, and frozen in liquid nitrogen. Six micrometer-thick

sections were cut and mounted to poly-lysine-coated glass slides.

Mouse corneal sections were fixed for 10 minutes in 4% paraformaldehyde. Slides blocked with 10 mM sodium phosphate buffer containing 2% BSA for 1 hour at room temperature were then incubated with rabbit primary antibodies (Serpine1, Plau, Sigma-Aldrich Corp., St. Louis, MO, USA). This was followed by a secondary antibody, FITC anti-rabbit (1:100; Jackson ImmunoResearch Laboratories, West

FIGURE 2. Real-time PCR verification of uPA system gene expression in healing versus homeostatic CECs of NL and DM mice ([A] Serpine1, [B] Plau, [C] Plaur). Corneal epithelial cells were collected from nondiabetic (NL) and STZ diabetic (DM) mouse corneas during epithelium-debridement or from the wound bed 24 hours after wounding (24h) (Fig. 1) and were subjected to real-time PCR analysis. The fold increase over that of the naïve corneas (value 1) is the mean $\pm$ SD of three samples ($n = 3$) for each condition with β -actin as the internal control for normalization. The results are representative of two independent experiments. * $P < 0.05$; ** $P < 0.01$ (one-way ANOVA).

FIGURE 3. Western blotting analysis of Serpine1 and Plau expression in healing versus homeostatic CECs of NL and DM mice. The scraped CECs from NL and DM mouse corneas for creating a wound and from the wound bed 24 hours after wounding, as described in Figure 2, were processed for Western blotting with β -actin as internal control for protein loading. (A) Two samples for each condition were run on the same gel. After transfer, the membrane was probed first with Serpine1 (48 kDa), after stripping, with Plau (34 kDa), and then actin (42 kDa). (B) The band intensities were calculated after background-subtracting and normalized with actin intensity using Carestream Molecular Imaging Software. The fold increase over that of the normal, unwounded cornea (value 1) is the mean $\pm$ SD of three samples ($n = 2$, two corneas each) for each condition with β -actin as the internal control for normalization. The results are representative of two independent experiments (two each). * $P < 0.05$ (one-way ANOVA).

Grove, PA, USA). Slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) mounting media. Controls were similarly treated, but the primary antibody was replaced with rat or rabbit IgG. The sections were examined under a Nikon ECLIPSE 90i microscope (Nikon, Melville, NY, USA). The center of unwounded and the leading edge of healing corneas were photographed. The epifluorescence images were acquired using image-acquisition software with identical setting for a set of samples. For semi-quantitative analysis of Serpine1 and Plau expression at the tissue levels, the fluorescence intensity was calculated using ImageJ (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and the integrated densities were divided by the area with fluorescence intensity above the background.

Western Blot

For Western blot, epithelial cells of corneas with or without wound were collected and homogenized in 50 μ L radioimmunoprecipitation assay (RIPA) buffer, and the protein concentrations were determined with the protein assay kit. A total of 20 μ g protein was separated with 5% to 15% gradient SDS-PAGE and transferred to a 0.2- μ m pore size nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) that was stained with Serpine1, Plau, and MMP-3. The membrane was then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution; Jackson ImmunoResearch Laboratories). The bands were visualized with enhanced chemiluminescent (ECL, SuperSignal; Thermo Scientific, Pittsburgh, PA, USA) and the images were acquired using Kodak Image Station 4000R Pro (Carestream Health, Inc., Rochester, NY, USA). Band intensity was analyzed using Carestream Molecular Imaging Software (Carestream Health, Inc.).

Matrix Metalloproteinase-3 Activity Assay

Matrix metalloproteinase-3 activity of CECs with or without a wound was tested by MMP-3 activity kit from Abcam (ab112148; Abcam, Cambridge, MA, USA). The assay was performed using CEC extracts in a 96-well microtiter plate and the signal was read by a fluorescence microplate reader BioTek Synergy2 (Winooski, VT, USA) at Ex/Em = 485/528 nm.

Statistical Analysis

Data were presented as mean $\pm$ SD. Statistical differences among three or more groups were first identified using one-way ANOVA, followed by Student's *t*-test for pairwise comparison. Differences were considered statistically significant at P less than 0.05.

RESULTS

Mouse STZ Diabetic Model and Delayed Epithelial Wound Healing

Our previous studies used STZ-induced rats as type 1 and GK rats with Wistar rats as the control for type 2 models of DM.^{20,43} As many more molecular reagents as well as genetically modified animals were available, we adapted a B6 mouse model of DM using low-dose STZ induction protocol for mice. However, average blood sugar levels were lower for mice (>400 dg/mL) than STZ rats (>500 dg/mL). At 10 weeks of hyperglycemia, we performed the wound-healing assay using an epithelial debridement wound model and found that at 24 hpw, the remaining wounds were significantly larger in DM compared with NL corneas of age-matched B6 mice, indicating a delay in corneal epithelia wound healing in DM B6 mice (Fig.

FIGURE 4. Serpine1 and Plau expression and distribution in wound-healing corneas of NL and DM mice assessed by immunohistochemistry. B6 and STZ B6 mouse corneas were wounded as described in Figure 1. At 24 hpw, corneas with remaining wounds were excised and snap-frozen in OCT compound, followed by sectioning and immunostaining with antibodies against Serpine1 or Plau. Unwounded corneas of NL and DM mice also were stained; DAPI was used to stain nuclei. (A) The photographs were taken at the center of unwounded cornea or the leading edge of healing corneas with the same exposure time and other settings. (B) The fluorescence intensity was semiquantitatively analyzed using ImageJ. The fold increase over that of the normal, unwounded cornea (value 1) is the mean $\pm$ SD of three samples ($n = 3$) for each condition. The results are representative of two independent experiments. * $P < 0.05$; ** $P < 0.01$ (one-way ANOVA).

1). This delayed epithelial wound closure in diabetic mice was observed.

Differential Expression and Distribution of Serpine1 in DM Healing Corneas

The Table shows cDNA array results of the genes involved in plasminogen activation.²⁰ Although the levels of plasminogen and Plat (tPA) remained unchanged, the expressions of Plau (uPA), Plaur (uPA receptor), and Serpine1 (PAI-1) were upregulated in response to wounding in STZ diabetic rats. Moreover, the wound-induced expressions of these three genes were inhibited by hyperglycemia in diabetic rats. To verify their expression patterns, real-time PCR was performed using the isolated mouse CECs (Fig. 2). Wounding induced approximately 60-fold increases in Serpine1 and approximately 10-fold in Plau and 40-fold in Plaur mRNA levels in normoglycemia mouse corneas; these increases were significantly suppressed to different extents in healing mouse CECs of STZ mice.

Figure 3 shows Western blotting analysis of the collected mouse CECs, with two samples for each condition (from four corneas). In unwounded corneas, Serpine1 and Plau were detected in both NL and DM CECs. In healing corneas, elevated expressions of both Serpine1 and Plau were apparent in NL,

but not in DM CECs (Fig. 3A). Image analysis revealed that in unwounded corneas, the levels of Serpine1, but not Plau in DM CECs were higher than that in NL CECs. On the other hand, epithelial wounding induced significant increases of Serpine1 and Plau at the protein levels in NL but not DM CECs.

The expression and distribution of Serpine1 and its target Plau also were assessed using immunohistochemistry in the control and STZ mice (Fig. 4). Low or undetectable levels of Serpine1 or Plau staining were observed in unwounded corneas. In healing epithelia, Serpine1 or Plau was stained intensively at the leading edge of NL corneas, and their staining in DM cornea was less intensive. A few infiltrated cells and/or stroma fibroblasts in healing corneas also were observed. Image analysis with ImageJ, a semi-quantitative assessment, revealed that although there were no detectable differences in relative fluorescence intensity per unit in unwounded corneas between NL and DM mice, much higher staining levels for both Serpine1 and Plau were observed at the leading edges of both healing NL and DM corneas, compared with that of unwounded corneas. Moreover, consistently stronger staining for Serpine1 or Plau in the epithelial sheets was observed in the NL corneas, compared with that in the DM cornea.

Taken together, these data revealed that PAI-1/Serpine1 and other components of the uPA system are upregulated in

response to wounding, and their upregulation is lessened in diabetic CECs.

Serpine1 Accelerates Delayed Epithelial Wound Healing in Diabetic Corneas

Serpine1 has been shown to delay skin wounding in vivo but promote keratinocyte migration wound healing in cultured cells.^{44–46} As corneal epithelial wound healing is primarily a reepithelialization process, we speculated Serpine1 and/or the uPA proteolytic system would have beneficial effects on corneal wound closure. To test this hypothesis, we used two approaches: antibody-mediated functional blocking in normal and diabetic cornea and Serpine1 supplementation in diabetic corneas (Fig. 5). Subconjunctival injection of Serpine1 neutralizing antibody significantly delayed epithelial wound closure in normal corneas and exhibited no significant effects on diabetic corneas, presumably due to the lack of Serpine1 activity in diabetic corneas. Supplementing Serpine1 by the subconjunctival injection of recombinant protein 4 hours before epithelial debridement in diabetic B6 mouse corneas accelerated epithelial wound healing to a level comparable to normal corneas (Fig. 5).

Plau and MMP-3 Expressions Were Altered in a Serpine1-Related Manner

Similar expression patterns of Serpine1, Plau, and Plaur suggest that Serpine1 functions through the Plau complex. Having shown that the alteration of Serpine1 levels resulted in the change of the rate of epithelial wound closure, we next investigated whether Plau levels were modulated by Serpine1. The CECs scraped from aforementioned corneas were subjected to real-time PCR (Fig. 6). Serpine1 neutralization significantly dampened wound-induced expression of Plau in NL cornea, whereas exogenously added Serpine1 increased Plau expression in DM corneas (Fig. 6).

Our cDNA array study revealed that MMP-3 was the most highly inducible gene among all MMPs assessed in CECs in response to wounding in NL, but not DM corneas.²⁰ Serpine1, in the form of the complex with tPA, was shown to trigger the induction of MMP-3 that potentially induces neurovascular disruption after neurotrauma.⁴⁷ As such, we also assessed the expression of MMP-3 in Serpine1-manipulated corneas. Real-time PCR confirmed cDNA array data. Similar to Plau, the robust upregulation of MMP-3 in healing CECs of NL corneas was suppressed by Serpine1 neutralization, whereas exogenously added Serpine1 significantly increased MMP-3 expression in diabetic corneas (Fig. 6). To assess if the elevated MMP-3 expression is active, we performed an in vitro enzyme assay of CECs using MMP-3-specific substrate. Figure 7 shows elevated MMP-3 activity in healing CECs of NL corneas and its suppression in DM corneas. Controlling Serpine1 expression affected MMP-3 activities in a manner similar to its expression at the mRNA levels in NL and DM corneas.

DISCUSSION

In this study, we used the data generated from our genome-wide cDNA microarray study²⁰ and focused on one gene, *Serpine1*, and its associated uPA proteolytic pathway because of the known functions and the altered expression pattern in healing DM versus NL CECs. We confirmed the differential expression patterns of Serpine1 along with its major target gene Plau and cell-surface receptor for PAI-1-uPA complex (Plaur) in DM mouse corneas and showed their robust expressions at migratory epithelial sheets in normal and, to a

FIGURE 5. Effects of Serpine1 on corneal epithelial wound healing in NL and DM mice. Serpine1 neutralizing antibody (2.5 $\mu\text{g}/5 \mu\text{L}$), with the same dosage of rabbit IgG as the control, were injected into subconjunctival space of NL and STZ B6 mice or 500 ng/5 μL recombinant Serpine1 (Millipore, Billerica, MA, USA) to STZ B6 mice 4 hours before wounding. A 1.5-mm wound was allowed to heal for 24 hours, fluorescence stained, and photographed (A). These figures are representative of six corneas per condition from two independent experiments. (B) The wound sizes were presented as the average number of pixels of the fluorescent-stained area (Fig. 1). The error bars represent the SEM ($n = 6$) and indicated P values were generated using paired Student's t -test. Two independent experiments were performed.

much less extent, in diabetic healing corneal CECs. Our results revealed that neutralization of Serpine1 activity resulted in a delay of epithelial wound closure in normal, nondiabetic mouse corneas, but exhibited minimal effects on diabetic corneas. On the other hand, treating diabetic corneas with recombinant Serpine1 accelerated epithelial wound closure in STZ B6 mice. In addition to Plau, our results for the first time showed that Serpine1 activity affected not only the expression

FIGURE 6. Effects of Serpine1 levels on Plau and MMP-3 expressions in healing versus homeostatic CECs of NL and DM mice. Corneal endothelial cells were scraped off from NL and DM mouse corneas for creating a wound (0 hour) from the wound bed 24 hpw as described in Figure 5. The collected CECs were subjected to real-time

but also the enzymatic activity of MMP-3 in healing CECs in vivo. The hyperglycemia-suppressed expressions of Serpine1 and Plau also were observed in injured human DM cornea compared with the control, nondiabetic one. Taken together, our study suggests that the dysregulation of Serpine1 expression and/or defects in the uPA proteolytic system may contribute to the delayed epithelial wound healing in diabetic corneas and Serpine1 or its derived peptides might be used as a therapeutic reagent, alone or as an adjuvant therapy, to accelerate wound closure in patients with diabetes.

Although tPA proteolytic system is known to be related to intravascular fibrinolysis and thrombolysis, the uPA is associated with the processes involving pericellular proteolysis required for cell migration and adhesion as the activation of pro-uPA is accelerated by its binding to a specific cell membrane receptor, uPAR.⁴⁸⁻⁵¹ The findings that Serpine1, PLAU, and PLAUR have similar expression patterns in homeostatic and healing CECs of normal and diabetic mice indicate that the uPA proteolytic system is activated in response to wounding and plays a role in mediating wound healing. Serpine1 was found to be elevated in repairing/migrating epithelial cells and its knockdown impairs epithelial wound repair.^{46,52-55} It has been known to play a key role in corneal wound healing.^{55,56} In humans, complete Serpine1 deficiency results in life-threatening hemorrhage and prolonged wound healing.⁵⁷ In this study, we reported that the mRNA levels of Serpine1, Plau, and Plaur were all increased in healing epithelial cells compared with homeostatic CEC in B6 mouse corneas. Moreover, abundant staining of Serpine1 and Plau was detected at the leading edge of migratory epithelial sheet. Several studies have reported the distribution of Serpine1 and Serpine1-Plau-Plaur complex at the leading edge of in vitro wounds.^{46,53,58,59} Hence, SERPINE1 may play a role in wound healing by modulating pericellular matrix restructuring and cell migration in the cornea. Although the expression levels of Plg (tPA) and Plat were not changed, further study of their role and their interplay with the uPA system is warranted.

Previous studies by Saghizadeh et al.⁶⁰ revealed elevated expressions of cathepsin F (a member of the papain family of cysteine proteinases), MMP-10, and MMP-3⁶¹ in unwounded as well as MMP-10 in healed¹² human diabetic corneas. Silencing of MMP-10 and cathepsin F resulted in enhanced wound healing in diabetic organ-cultured human corneas.²⁴ Our study is the first to show that the wound-induced upregulation of Serpine1, PLAU and PLAUR were suppressed by hyperglycemia. Diabetic patients show elevated levels of PAI-1 in plasma.^{62,63} The higher PAI-1 plasma level was found to be independently associated with a lower risk of retinopathy but a higher risk of coronary heart disease in type 2 diabetes.³⁸ In animal studies, renal PAI-1 gene expression is upregulated in both type 1 and type 2 diabetic rats.⁶⁴ Moreover, diabetic rats exhibited high levels of PAI-1, uPA, and uPAR mRNAs and type IV collagen protein in glomeruli, mainly in mesangial cells.⁶⁵ Epigenetic studies revealed a glucose-independent persistence of PAI-1 gene expression and H3K4 tri-methylation in type 1 diabetic mouse endothelium, suggesting the potential involvement of PAI-1 in metabolic memory.⁶⁶ Our epithelial wound-healing models reflect acute induction and activation of the uPA proteolytic system in migratory epithelial cells. Unlike the unstimulated condition, this robust upregulation observed in

PCR. The data were presented as mean fold change in the expression of indicated gene over nondiabetic, 0-hour CECs. The error bars represent the SEM ($n = 3$) and indicated P values were generated using paired Student's t -test. Two independent experiments were performed.

FIGURE 7. Effects of Serpine1 levels on MMP-3 enzymatic activity in healing versus homeostatic CECs of NL and DM mice. Corneal endothelial cells were scraped off from NL and DM mouse corneas for creating a wound (0 hour) and from the wound bed 24 hpw as described in Figure 5. These collected CECs were lysed in RIPA buffer and the cell extracts after debris removal were analyzed by MMP-3 Activity kit from Abcam using 50 μ g total proteins from each sample. The data were presented as mean fold change in MMP-3 enzymatic activities over nondiabetic, 0-hour CECs as 1. The error bars represent the SEM ($n = 3$) and indicated P values were generated using paired Student's t -test. Two independent experiments were performed.

normoglycemia mice was greatly reduced in the diabetic cornea, consistent with much-delayed epithelial wound closure. Thus, although the elevated basal expression of PAI-1 or activity of the uPA proteolytic system may be detrimental to the homeostasis of different tissues, insufficient expression of these genes contributes to the impairment of wound healing commonly observed in the diabetic cornea.

Serpine1 expression has long been recognized as TGF β 1-mediated.^{67,68} Our previous study revealed that although the expression of both TGF β 1 and β 3 were greatly elevated in the epithelial cells of normal, nondiabetic cornea in response to wounding, the elevated expression of TGF β 3, but not β 1, was suppressed by hyperglycemia.²⁰ This raises the possibility that the expression of Serpine1 in healing CECs is regulated by TGF β 3. Alternatively, cumulated activity of TGF β isoforms is a determining factor for Serpine1 expression. Interestingly, using Serpine1 knockout mice, deficiency of Serpine1 was found to impair TGF β 1 expression in the diabetic mouse kidney cortex.⁶⁹ Serpine1 was found to regulate TGF β 1 expression by binding to uPA and uPAR and activating MAPK.⁶⁹ Hence, further study to characterize the influences of TGF β and Serpine1 on each other in the regulation of gene expression during wound healing is warranted.

An early study of the mitogen-activated protein kinase (MEK) kinase 1 (MEKK1) revealed that MEKK1 mediated extracellular matrix (ECM) homeostasis, epithelial cell migration, and wound reepithelialization through Serpine1 and MMP-3 in mouse corneas.⁵⁶ Interesting, our study revealed a correlation between the levels of Serpine1 and MMP-3 expression and activity. In the literature, increases in the mRNA and protein levels of uPA and MMP-3 were observed in the endometrium of women with endometriosis.⁷⁰ Moreover, overexpression of uPA in cultured hepatic stellate cells resulted in a great overactivation of MMP3.⁷¹ On the other hand, MMP3 was found to inactivate Serpine1 by specific proteolysis.⁷² Our study is the first to suggest a role for uPA-PAI-1-MMP-3 axis in promoting epithelial wound repair. Modulating uPA proteolytic system or downstream MMP-3 induction may provide viable

therapeutic strategies to restore epithelial wound healing in diabetic corneas.

Finally, the significance of these findings was further strengthened by the confirmation of greatly elevated expression of Serpine1 and Plau in healing epithelial sheets of normal, but not diabetic, corneas, suggesting clinic relevance of the mouse model of diabetic corneal wound healing. Demonstrating that exogenous Serpine1 facilitates corneal epithelial wound closure in diabetic corneas suggests the potential clinical use of the protein or its derived peptide, EEIIMD^{73,74} or THR-18,⁷⁵ as a therapeutic reagent to treat diabetic keratopathy and delayed epithelial wound healing in diabetic patients.

Acknowledgments

Supported by grants from the National Institutes of Health (R01EY10869 and EY17960) and P30-EY-04078 (National Eye Institute core to WSUSOM, PI LDH) and an unrestricted grant from Research to Prevent Blindness (Department of Ophthalmology, Wayne State University).

Disclosure: **H. Sun**, None; **X. Mi**, None; **N. Gao**, None; **C. Yan**, None; **F.-S. Yu**, None

References

- Clark EA, Grabstein KH, Gown AM, et al. Activation of B lymphocyte maturation by a human follicular dendritic cell line, FDC-1. *J Immunol*. 1995;155:545-555.
- Threatt J, Williamson JF, Huynh K, Davis RM. Ocular disease, knowledge and technology applications in patients with diabetes. *Am J Med Sci*. 2013;345:266-270.
- Kaji Y. Prevention of diabetic keratopathy. *Br J Ophthalmol*. 2005;89:254-255.
- Pflugfelder SC. Is autologous serum a tonic for the ailing corneal epithelium? *Am J Ophthalmol*. 2006;142:316-317.
- Bikbova G, Oshitari T, Tawada A, Yamamoto S. Corneal changes in diabetes mellitus. *Curr Diabetes Rev*. 2012;8:294-302.

6. Chikama T, Wakuta M, Liu Y, Nishida T. Deviated mechanism of wound healing in diabetic corneas. *Cornea*. 2007;26:S75-S81.
7. Simpson RG, Moshirfar M, Edmonds JN, Christiansen SM. Laser in-situ keratomileusis in patients with diabetes mellitus: a review of the literature. *Clin Ophthalmol*. 2012;6:1665-1674.
8. Friend J, Ishii Y, Thoft RA. Corneal epithelial changes in diabetic rats. *Ophthalmic Res*. 1982;14:269-278.
9. Azar DT, Spurr-Michaud SJ, Tisdale AS, Gipson IK. Altered epithelial-basement membrane interactions in diabetic corneas. *Arch Ophthalmol*. 1992;110:537-540.
10. McDermott AM, Xiao TL, Kern TS, Murphy CJ. Non-enzymatic glycation in corneas from normal and diabetic donors and its effects on epithelial cell attachment in vitro. *Optometry*. 2003;74:443-452.
11. Ljubimov AV, Alba SA, Burgeson RE, et al. Extracellular matrix changes in human corneas after radial keratotomy. *Exp Eye Res*. 1998;67:265-272.
12. Kabosova A, Kramerov AA, Aoki AM, Murphy G, Zieske JD, Ljubimov AV. Human diabetic corneas preserve wound healing, basement membrane, integrin and MMP-10 differences from normal corneas in organ culture. *Exp Eye Res*. 2003;77:211-217.
13. Zagon IS, Sassani JW, McLaughlin PJ. Insulin treatment ameliorates impaired corneal reepithelialization in diabetic rats. *Diabetes*. 2006;55:1141-1147.
14. Wakuta M, Morishige N, Chikama T, Seki K, Nagano T, Nishida T. Delayed wound closure and phenotypic changes in corneal epithelium of the spontaneously diabetic Goto-Kakizaki rat. *Invest Ophthalmol Vis Sci*. 2007;48:590-596.
15. Inoue K, Okugawa K, Amano S, et al. Blinking and superficial punctate keratopathy in patients with diabetes mellitus. *Eye*. 2005;19:418-421.
16. Rehany U, Ishii Y, Lahav M, Rumelt S. Ultrastructural changes in corneas of diabetic patients: an electron-microscopy study. *Cornea*. 2000;19:534-538.
17. Saini JS, Khandalavla B. Corneal epithelial fragility in diabetes mellitus. *Can J Ophthalmol*. 1995;30:142-146.
18. Schultz RO, Van Horn DL, Peters MA, Klewin KM, Schutten WH. Diabetic keratopathy. *Trans Am Ophthalmol Soc*. 1981;79:180-199.
19. Xu K, Yu FS. Impaired epithelial wound healing and EGFR signaling pathways in the corneas of diabetic rats. *Invest Ophthalmol Vis Sci*. 2011;52:3301-3308.
20. Bettahi I, Sun H, Gao N, et al. Genome-wide transcriptional analysis of differentially expressed genes in diabetic, healing corneal epithelial cells: hyperglycemia-suppressed TGF β 3 expression contributes to the delay of epithelial wound healing in diabetic corneas. *Diabetes*. 2014;63:715-727.
21. Saghizadeh M, Kramerov AA, Yu FS, Castro MG, Ljubimov AV. Normalization of wound healing and diabetic markers in organ cultured human diabetic corneas by adenoviral delivery of c-Met gene. *Invest Ophthalmol Vis Sci*. 2010;51:1970-1980.
22. McLaughlin PJ, Zagon IS. The opioid growth factor-opioid growth factor receptor axis: homeostatic regulator of cell proliferation and its implications for health and disease. *Biochem Pharmacol*. 2012;84:746-755.
23. Funari VA, Winkler M, Brown J, Dimitrijevic SD, Ljubimov AV, Saghizadeh M. Differentially expressed wound healing-related microRNAs in the human diabetic cornea. *PLoS One*. 2013;8:e84425.
24. Saghizadeh M, Epifantseva I, Hemmati D, Ghiam CA, Brunken WJ, Ljubimov AV. Enhanced wound healing, kinase and stem cell marker expression in diabetic organ-cultured human corneas upon MMP-10 and cathepsin F gene silencing. *Invest Ophthalmol Vis Sci*. 2013;54:8172-8180.
25. Ghosh AK, Vaughan DE. PAI-1 in tissue fibrosis. *J Cell Physiol*. 2012;227:493-507.
26. Lyons RM, Gentry LE, Purchio AF, Moses HL. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol*. 1990;110:1361-1367.
27. Taipale J, Saharinen J, Keski-Oja J. Extracellular matrix-associated transforming growth factor-beta: role in cancer cell growth and invasion. *Adv Cancer Res*. 1998;75:87-134.
28. Deng GG, Martin-McNulty B, Sukovich DA, et al. Urokinase-type plasminogen activator plays a critical role in angiotensin II-induced abdominal aortic aneurysm. *Circ Res*. 2003;92:510-517.
29. Mazzieri R, Masiero L, Zanetta L, et al. Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. *EMBO J*. 1997;16:2319-2332.
30. Jankun J, Al-Senaidy A, Skrzypczak-Jankun E. Can inactivators of plasminogen activator inhibitor alleviate the burden of obesity and diabetes? (Review). *Int J Mol Med*. 2012;29:3-11.
31. Ha H, Oh EY, Lee HB. The role of plasminogen activator inhibitor 1 in renal and cardiovascular diseases. *Nat Rev Nephrol*. 2009;5:203-211.
32. Hertig A, Berrou J, Allory Y, et al. Type 1 plasminogen activator inhibitor deficiency aggravates the course of experimental glomerulonephritis through overactivation of transforming growth factor beta. *FASEB J*. 2003;17:1904-1906.
33. Lackie PM. Molecular portfolios: cells interacting with matrix in repairing airway epithelium. *Clin Exp Allergy*. 2008;38:1840-1843.
34. Tervo T, Tervo K, van Setten GB, Virtanen I, Tarkkanen A. Plasminogen activator and its inhibitor in the experimental corneal wound. *Exp Eye Res*. 1989;48:445-449.
35. Wang Z, Sosne G, Kurpakus-Wheeler M. Plasminogen activator inhibitor-1 (PAI-1) stimulates human corneal epithelial cell adhesion and migration in vitro. *Exp Eye Res*. 2005;80:1-8.
36. Hazra S, Stepps V, Bhatwadekar AD, et al. Enhancing the function of CD34(+) cells by targeting plasminogen activator inhibitor-1. *PLoS One*. 2013;8:e79067.
37. Brazionis L, Yau J, Rowley K, et al. Plasminogen activator inhibitor-1 (PAI-1) activity and retinal vascular calibre in type 2 diabetes. *Diabetes Res Clin Pract*. 2010;87:192-199.
38. Brazionis L, Rowley K, Jenkins A, Itsiopoulos C, O'Dea K. Plasminogen activator inhibitor-1 activity in type 2 diabetes: a different relationship with coronary heart disease and diabetic retinopathy. *Arterioscler Thromb Vasc Biol*. 2008;28:786-791.
39. O'Reilly N, Gallagher C, Reddy Katikireddy K, Clynes M, O'Sullivan F, Kavanagh K. Demodex-associated *Bacillus* proteins induce an aberrant wound healing response in a corneal epithelial cell line: possible implications for corneal ulcer formation in ocular rosacea. *Invest Ophthalmol Vis Sci*. 2012;53:3250-3259.
40. Seet LF, Tong L, Su R, Wong TT. Involvement of SPARC and MMP-3 in the pathogenesis of human pterygium. *Invest Ophthalmol Vis Sci*. 2012;53:587-595.
41. Sashindranath M, Sales E, Daglas M, et al. The tissue-type plasminogen activator-plasminogen activator inhibitor 1 complex promotes neurovascular injury in brain trauma: evidence from mice and humans. *Brain*. 2012;135:3251-3264.
42. Yin J, Huang J, Chen C, Gao N, Wang F, Yu FS. Corneal complications in streptozocin-induced type I diabetic rats. *Invest Ophthalmol Vis Sci*. 2011;52:6589-6596.
43. Wang F, Gao N, Yin J, Yu FS. Reduced innervation and delayed re-innervation after epithelial wounding in type 2 diabetic Goto-Kakizaki rats. *Am J Pathol*. 2012;181:2058-2066.
44. Chan JC, Duszczyk DA, Castellino FJ, Ploplis VA. Accelerated skin wound healing in plasminogen activator inhibitor-1-deficient mice. *Am J Pathol*. 2001;159:1681-1688.
45. Providence KM, Higgins SP, Mullen A, et al. SERPINE1 (PAI-1) is deposited into keratinocyte migration "trails" and required

- for optimal monolayer wound repair. *Arch Dermatol Res*. 2008;300:303–310.
46. Providence KM, Higgins PJ. PAI-1 expression is required for epithelial cell migration in two distinct phases of in vitro wound repair. *J Cell Physiol*. 2004;200:297–308.
 47. Sashindranath M, Sales E, Daglas M, et al. The tissue-type plasminogen activator-plasminogen activator inhibitor 1 complex promotes neurovascular injury in brain trauma: evidence from mice and humans. *Brain*. 2012;135:3251–3264.
 48. Mekkiwaty AH, Pourgholami MH, Morris DL. Involvement of urokinase-type plasminogen activator system in cancer: an overview. *Med Res Rev*. 2014;34:918–956.
 49. Li WY, Chong SS, Huang EY, Tuan TL. Plasminogen activator/plasmin system: a major player in wound healing? *Wound Repair Regen*. 2003;11:239–247.
 50. Choong PF, Nadesapillai AP. Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. *Clin Orthop Relat Res*. 2003;415:S46–S58.
 51. Nicholl SM, Roztocil E, Davies MG. Plasminogen activator system and vascular disease. *Curr Vasc Pharmacol*. 2006;4:101–116.
 52. Stewart CE, Sayers I. Urokinase receptor orchestrates the plasminogen system in airway epithelial cell function. *Lung*. 2013;191:215–225.
 53. Providence KM, Higgins SP, Mullen A, et al. SERPINE1 (PAI-1) is deposited into keratinocyte migration “trails” and required for optimal monolayer wound repair. *Arch Dermatol Res*. 2008;300:303–310.
 54. Maquerlot F, Galiacy S, Malo M, et al. Dual role for plasminogen activator inhibitor type 1 as soluble and as matricellular regulator of epithelial alveolar cell wound healing. *Am J Pathol*. 2006;169:1624–1632.
 55. Stevens PT, Kicic A, Sutanto EN, Knight DA, Stick SM. Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1. *Clin Exp Allergy*. 2008;38:1901–1910.
 56. Deng M, Chen WL, Takatori A, et al. A role for the mitogen-activated protein kinase kinase 1 in epithelial wound healing. *Mol Biol Cell*. 2006;17:3446–3455.
 57. Iwaki T, Tanaka A, Miyawaki Y, et al. Life-threatening hemorrhage and prolonged wound healing are remarkable phenotypes manifested by complete plasminogen activator inhibitor-1 deficiency in humans. *J Thromb Haemost*. 2011;9:1200–1206.
 58. Dach K, Zovko J, Hogardt M, et al. Bacterial toxins induce sustained mRNA expression of the silencing transcription factor klf2 via inactivation of RhoA and RhoGDI 1. *Infect Immun*. 2009;77:5583–5592.
 59. Providence KM, Kutz SM, Staiano-Coico L, Higgins PJ. PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. *J Cell Physiol*. 2000;182:269–280.
 60. Saghizadeh M, Kramerov AA, Tajbakhsh J, et al. Proteinase and growth factor alterations revealed by gene microarray analysis of human diabetic corneas. *Invest Ophthalmol Vis Sci*. 2005;46:3604–3615.
 61. Saghizadeh M, Brown DJ, Castellon R, et al. Overexpression of matrix metalloproteinase-10 and matrix metalloproteinase-3 in human diabetic corneas: a possible mechanism of basement membrane and integrin alterations. *Am J Pathol*. 2001;158:723–734.
 62. McGill JB, Schneider DJ, Arfken CL, Lucore CL, Sobel BE. Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. *Diabetes*. 1994;43:104–109.
 63. Schneider DJ, Sobel BE. PAI-1 and diabetes: a journey from the bench to the bedside. *Diabetes Care*. 2012;35:1961–1967.
 64. Hagiwara H, Kaizu K, Uriu K, et al. Expression of type-1 plasminogen activator inhibitor in the kidney of diabetic rat models. *Thromb Res*. 2003;111:301–309.
 65. Kenichi M, Masanobu M, Takehiko K, et al. Renal synthesis of urokinase type-plasminogen activator, its receptor, and plasminogen activator inhibitor-1 in diabetic nephropathy in rats: modulation by angiotensin-converting-enzyme inhibitor. *J Lab Clin Med*. 2004;144:69–77.
 66. Takizawa F, Mizutani S, Ogawa Y, Sawada N. Glucose-independent persistence of PAI-1 gene expression and H3K4 tri-methylation in type 1 diabetic mouse endothelium: implication in metabolic memory. *Biochem Biophys Res Commun*. 2013;433:66–72.
 67. Samarakoon R, Higgins CE, Higgins SP, Higgins PJ. TGF-beta1-induced expression of the poor prognosis SERPINE1/PAI-1 gene requires EGFR signaling: a new target for anti-EGFR therapy. *J Oncol*. 2009;2009:342391.
 68. Johnsen SA, Subramaniam M, Janknecht R, Spelsberg TC. TGFbeta inducible early gene enhances TGFbeta/Smad-dependent transcriptional responses. *Oncogene*. 2002;21:5783–5790.
 69. Nicholas SB, Aguiniga E, Ren Y, et al. Plasminogen activator inhibitor-1 deficiency retards diabetic nephropathy. *Kidney Int*. 2005;67:1297–1307.
 70. Ramon L, Gilabert-Estelles J, Castello R, et al. mRNA analysis of several components of the plasminogen activator and matrix metalloproteinase systems in endometriosis using a real-time quantitative RT-PCR assay. *Hum Reprod*. 2005;20:272–278.
 71. Gonzalez-Cuevas J, Bueno-Topete M, Armendariz-Borunda J. Urokinase plasminogen activator stimulates function of active forms of stromelysin and gelatinases (MMP-2 and MMP-9) in cirrhotic tissue. *J Gastroenterol Hepatol*. 2006;21:1544–1554.
 72. Lijnen HR, Arza B, Van Hoef B, Collen D, Declercq PJ. Inactivation of plasminogen activator inhibitor-1 by specific proteolysis with stromelysin-1 (MMP-3). *J Biol Chem*. 2000;275:37645–37650.
 73. Zhang L, Strickland DK, Cines DB, Higazi AA. Regulation of single chain urokinase binding, internalization, and degradation by a plasminogen activator inhibitor 1-derived peptide. *J Biol Chem*. 1997;272:27053–27057.
 74. Armstead WM, Riley J, Kiessling JW, Cines DB, Higazi AA. PAI-1-derived peptide EEIIMD prevents impairment of cerebrovasodilation by augmenting p38 MAPK upregulation after cerebral hypoxia/ischemia. *Am J Physiol Heart Circ Physiol*. 2010;299:H76–H80.
 75. Krakovsky M, Polianski V, Nimrod A, Higazi A, Leker RR, Lamensdorf I. THR-18, a 18-mer peptide derived from PAI-1, is neuroprotective and improves thrombolysis by tPA in rat stroke models. *Neurol Res*. 2011;33:983–990.