Fibroblast growth factor-2 did not restore plasminogen system activity in endothelial cells on glycated collagen

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ABSTRACT

People with diabetes experience morbidity and mortality from unregulated microvascular remodeling, which may be linked to hyperglycemia. Elevated glucose leads to extracellular matrix collagen glycation, which delays endothelial capillary-like tube formation in vitro. Glucose also increases endothelial cell fibroblast growth factor-2 (FGF-2) release and extracellular matrix storage, which should increase tube formation. In this study, we determined if FGF-2 could restore plasminogen system activity and angiogenic function in endothelial cells on glycated collagen. Human umbilical vein endothelial cells cultured on native or glycated collagen substrates were stimulated with FGF-2. Plasminogen system activity, cell migration, and capillary-like tube formation were measured, along with plasminogen system protein and mRNA levels. Glycated collagen decreased endothelial cell plasminogen system activity, cell migration, and tube length. FGF-2 did not restore plasminogen system activity or tube formation in cells on glycated collagen, despite decreasing plasminogen activator inhibitor-1 (PAI-1) protein level. We now show that PAI-1 binds to glycated collagen, which may localize PAI-1 to the extracellular matrix. These data suggest that FGF-2 may not restore angiogenic functions in endothelial cells on glycated collagen due to PAI-1 bound to glycated collagen.

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1. Introduction

Diabetic hyperglycemia is implicated in diseases of increased angiogenesis (e.g., diabetic nephropathy and retinopathy) as well as diseases of decreased angiogenesis (e.g., diabetic neuropathy and impaired wound healing) [1–4]. In angiogenesis, endothelial cells must degrade the extracellular matrix so that they can migrate and invade into the surrounding tissue, where they form capillary tubes [5]. Glucose modifies the extracellular matrix in ways that could both increase and decrease angiogenesis. Glucose reacts with amino protein groups to glycate collagen via the Maillard reaction, which enhances collagen cross-linking, makes it more resistant to proteolysis, and inhibits angiogenesis [6–10]. We previously showed that extracellular matrix from endothelial cells cultured in high glucose contains more fibroblast growth factor-2 (FGF-2), and retinal tissue from diabetic patients with proliferative retinopathy showed increased FGF-2 [11,12]. FGF-2 promotes angiogenesis in part by increasing extracellular matrix proteolysis [13].

Extracellular matrix proteolysis in angiogenesis is driven by the plasminogen system and matrix metalloproteinases [14]. In the plasminogen system, urokinase plasminogen activator (uPA) is the primary activator of pericellular matrix proteolysis [15]. Cells secrete uPA as a single chain pro-enzyme (scuPA) [16]. Secreted scuPA binds to its cell surface receptor (uPAR), where plasmin cleaves it to the active two-chain uPA form. uPA then cleaves plasminogen to plasmin, which either directly breaks down matrix components or activates matrix metalloproteinases [17]. Plasminogen activator inhibitor-1 (PAI-1) is the primary uPA inhibitor [18]. Cells secrete PAI-1 in active form but it rapidly decays into its latent form unless it binds to multimeric vitronectin in plasma, platelets, or extracellular matrix [19]. Active PAI-1 binds to and inactivates receptor bound uPA, preventing proteolysis [20]. The uPA:PAI-1 balance is postulated to break down enough of the extracellular matrix to enable cell invasion yet still maintain adequate matrix stability so that cells can adhere to and migrate along the matrix.

Both glycated collagen and FGF-2 impact the plasminogen system, albeit in opposite directions. Chen et al. showed delayed capillary cord branching in human umbilical vein endothelial cells on glycated as compared to native collagen gels, which was ascribed to elevated PAI-1 [10]. Elevated PAI-1 is prevalent in patients with metabolic syndrome, and both diabetic animals and humans showed increased PAI-1 in the arterial wall [21–23]. Thus glycated collagen shifts the plasminogen system balance in the anti-angiogenic direction by increasing PAI-1. FGF-2 increases uPA and PAI-1.
at both the transcriptional and translational levels [24,25]. However, FGF-2 stimulates more uPA than PAI-1. Thus FGF-2 moves the plasminogen system in a pro-angiogenic direction.

In this study, we investigated whether FGF-2 could restore angiogenic functions in endothelial cells on glycated collagen by activating the plasminogen system. Endothelial cells were cultured on native or glycated collagen with or without FGF-2. We now show that FGF-2 did not restore angiogenic functions in cells on glycated collagen despite decreasing PAI-1, perhaps due to increased PAI-1 binding to glycated collagen.

2. Materials and methods

2.1. Cell culture and collagen glycation

Human umbilical vein endothelial cells (HUVEC, passages 4–9; Lonza) were maintained in low glucose (5.5 mM) Endothelium Growth Medium (EGM-2; Lonza) supplemented with 5% fetal bovine serum (FBS; Hyclone), 1% penicillin–streptomycin (Gibco), and 1% glutamine (Gibco). HUVEC were selected because they are widely used for in vitro endothelial cell studies, show a robust FGF-2 response, and react with human plasminogen system proteins and antibodies. In addition, HUVEC were used in the original paper in which glycated collagen delayed endothelial cell capillary-like cord branching [10]. Collagen was glycated by incubating 100 μg/ml collagen type I (BD Biosciences) with 500 mM D-glucose-6-phosphate (G6P; Sigma) in phosphate buffered saline (PBS) at 37 °C for 4 weeks. Collagen glycation was validated via autofluorescence and decreased collagenase digestion as described previously [26]. Multimeric vitronectin was created by incubating 1 mg/ml native vitronectin (Molecular Innovations) with 6 M urea (Sigma) in 1× tris-buffered saline (TBS) for 1 h at 37 °C. After dialysis in 1× TBS for 18 h, multimeric vitronectin was collected and stored at −80 °C until use. Cell culture substrates were coated with 50 μg/ml native or glycated collagen (BD Biosciences) overnight at 4 °C. After washing, 25,000 cells/cm² were seeded on these substrates in Endothelial Basal Medium (EBM-2; Lonza) supplemented with 5% FBS and allowed to attach for 48 h EBM-2, which is EGM-2 without growth factors and cytokines, was used for all experiments to enable measurement of FGF-2 effects.

2.2. uPA activity

Chromozym PL was used to determine uPA activity in cell extracts. In our assay, uPA cleaved exogenous plasminogen to plasmin, which then cleaved Chromozyme PL into a residual peptide and 4-nitroaniline (405 nm). HUVEC were cultured for 48 h on native

![Fig. 1](image-url) Glycated collagen decreased endothelial cell uPA activity, 3D migration, and tube length compared to native collagen. FGF-2 only partially abrogated this effect. (A) uPA activity was measured using Chromozym PL. HUVEC seeded on native and glycated collagen coated substrates for 48 h were stimulated with 50 ng/ml FGF-2 for 24 h. (B) 3D cell migration was measured using a Boyden chamber. HUVEC ± 50 ng/ml FGF-2 were added to a Transwell insert coated with 100 μg/ml native or glycated collagen. After 24 h, cells that migrated to the chamber bottom were labeled with Hoechst, imaged by fluorescent microscopy, and quantified with ImageJ. Samples were normalized to native collagen without FGF-2. (C) For tube formation, HUVEC were added to native and glycated collagen gels (4 mg/ml) ± FGF-2 (50 ng/ml). After 18 h, samples were imaged by phase contrast microscopy and tube length was analyzed. (D) Sample tube formation images, with tubes indicated by black arrows. *p < 0.01; **p < 0.01 glycated vs. native collagen. Each experiment was completed in triplicate and repeated three times.
and glycated collagen coated substrates as described. 50 ng/ml FGF-2 (PeproTech) was added to cells in fresh EBM-2 for 24 h. HUVEC were lysed in T/T buffer (60 mM Tris hydrochloride, 0.5% Triton X-100) for 5 min, after which cell extracts were centrifuged at 10,000 g for 10 min to remove insoluble material. A final solution of cell extract, 127 ng/mL Chromozym PL (Roche), and 67 µU/mL plasminogen (Roche) was mixed in a 96 well plate. Absorbance (405 nm) was measured for 24 h in an Infinite 200 PRO microplate reader (TECAN) maintained at 37 °C. The change in 4-nitroaniline absorbance at 405 nm is directly proportional to uPA enzymatic activity. Absorbance was plotted vs. time, and the linear region slope (ΔA/min) was used to calculate uPA activity via the following equation:

\[ \text{uPA Activity (U/ml)/A/min} = \frac{V}{v \cdot c \cdot d} \cdot (\Delta A/\text{min}) \]

where \(V\) is total volume (300 µl), \(v\) is cell extract volume (33 µl), \(c\) is absorbance coefficient for 4-nitroaniline \((1.4 \times 10^4 \text{mol}^{-1} \text{cm}^{-1})\), and \(d\) is light path (1 cm).

### 3. 3D migration

HUVEC 3D migration was assessed using a Boyden chamber assay. The bottom of a Transwell chamber (6.5 mm diameter, 8 µm pore size; Corning Costar) was coated with 100 µg/ml native or glycated collagen, which was allowed to gel for 1 h at room temperature. 150,000 cells were then added to the top of each chamber in EBM-2 with 5% FBS with and without FGF-2 (50 ng/ml). EBM-2 with 10% FBS was added to the bottom chamber. Samples were incubated for 24 h at 37 °C, after which cells remaining in the upper Transwell chamber were removed by swabbing. Cells that migrated to the chamber bottom were labeled with Hoechst (10 ng/ml; Invitrogen) for 30 min. 5 randomly selected areas per sample were imaged using an Olympus IX81 inverted fluorescent microscope. Cell number was quantified with ImageJ.

### 3.1. Tube formation

HUVEC tube formation was assessed on native or glycated collagen gels. Native collagen gels were prepared by incubating 50 µl 4 mg/ml collagen I at 37 °C for 1 h. Glycated collagen gels were prepared by incubating native collagen gels with 500 mM G6P for 4 weeks at 37 °C. 15,000 HUVEC were added to each gel in EBM-2 with 5% FBS with and without FGF-2 (50 ng/ml). Cells were incubated for 18 h at 37 °C, after which samples were imaged using a Nikon Eclipse TS100 phase contrast microscope (5 images per well). Tube length was manually analyzed using ImageJ by an objective technician with no knowledge of the coded images [27].

### 3.2. uPA, PAI-1, and uPAR protein and mRNA levels

uPA, PAI-1, and uPAR protein and mRNA levels were quantified by Western blot and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), respectively. HUVEC were cultured for 48 h on native or glycated collagen coated substrates as described, after which they were treated with 50 ng/ml FGF-2 for 2–24 h. For Western blot, cells were scraped off the surface in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM NaVO₄, 2 mM PMSF, 50 mM NaF, 10%...
glycated collagen did not alter uPA or PAI-1 mRNA, whereas FGF-2 treatment decreased PAI-1 mRNA on both native and glycated collagen. HUVEC seeded on native and glycated collagen coated substrates for 48 h were stimulated with 50 ng/ml FGF-2 for (A, B) 2 or (C, D) 24 h. One-step RT-PCR was performed using a LightCycler 480. (A, C) uPA and (B, D) PAI-1 mRNA levels were normalized to GAPDH for each condition. *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was completed in triplicate and repeated three times.

Fig. 3. Glycated collagen did not alter uPA or PAI-1 mRNA, whereas FGF-2 treatment decreased PAI-1 mRNA on both native and glycated collagen. HUVEC seeded on native and glycated collagen coated substrates for 48 h were stimulated with 50 ng/ml FGF-2 for (A, B) 2 or (C, D) 24 h. One-step RT-PCR was performed using a LightCycler 480. (A, C) uPA and (B, D) PAI-1 mRNA levels were normalized to GAPDH for each condition. *p < 0.05, **p < 0.01, ***p < 0.001. Each experiment was completed in triplicate and repeated three times.
was then replaced with 1.8 mM 2,2-azinobis(3-ethylbenzthia-
dine-6-sulfonic) acid and incubated for 30 min at 37 °C. Absor-
bance (405 nm) was quantified using a microplate reader.

3.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism and
Instat software. Samples were collected in triplicate and experi-
ments performed at least two times. Data are graphed as
mean ± standard deviation. Significance between two groups was
compared using Student’s t-test. Comparisons among multiple
groups were analyzed by two-way ANOVA with a Bonferroni post-
hoc test. *p-values are indicated in the figures by *p < 0.05, **p < 0.01, unless otherwise indicated.

4. Results and discussion

4.1. Endothelial cell response to FGF-2 on native and glycated
collagen

Glycated collagen was previously shown to delay endothelial
cell capillary cord branching, perhaps due to elevated PAI-1 [10].
We therefore measured whether FGF-2 could reverse glycated
collagen-induced changes in plasminogen activity and angiogenic
functions. HUVEC attachment and spreading were statistically si-
milar on both native and glycated collagen coated substrates (data
not shown). However, uPA activity was 35% lower in cells on gly-
cated compared to native collagen (Fig. 1A). While FGF-2 increased
uPA activity by 51% in cells on native collagen, it only increased
uPA activity by 22% in cells on glycated collagen. Cell proliferation
(assessed by cell counts) and 2D migration (assessed by cage
assay) increased with FGF-2 on both native and glycated collagen,
with no differences based on substrate composition (data not
shown). In contrast, collagen glycation did change endothelial cell
3D migration through a collagen coated Boyden chamber. 27%
fewer cells migrated through the Boyden chamber on glycated as
compared to native collagen. FGF-2 stimulation restored cell mi-
gration on glycated collagen back to native collagen levels (Fig. 1B).
Endothelial cell tube length was 48% lower for HUVEC on glycated
collagen gels as compared to native collagen gels. FGF-2 more than
doubled tube length on native collagen gels, but only increased
tube length 46% on glycated collagen gels (Fig. 1C; sample images
in Fig. 1D). Thus FGF-2 fully rescued 3D migration but not plas-
minogen system activity or capillary-like tube formation from
glycated collagen effects.

4.2. Glycated collagen and FGF-2 effects on plasminogen system
components

Plasminogen system activity derives from uPA binding to uPAR
and the uPA:PAI-1 balance. We therefore measured uPA, uPAR, and
PAI-1 protein levels by Western blot (Fig. 2A) to determine which
proteins contributed to decreased plasminogen system activity in
cells on glycated collagen. For the selected time points (48 h on
glycated collagen followed by 2, 6, 12, or 24 h of FGF-2 stimula-
tion), glycated collagen and FGF-2 only changed PAI-1 protein le-
vels at 24 h. HUVEC on glycated collagen showed more than
twice the PAI-1 protein as cells on native collagen, and FGF-2
brought the PAI-1 level down to that of cells on native collagen
(24 h data in Fig. 2B). FGF-2 had no effect on PAI-1 protein in cells
on native collagen, and neither glycated collagen nor FGF-2 af-
fected uPA or uPAR levels across all time points (24 h data in
Fig. 2C and D).

Fig. 4. PAI-1 bound to glycated collagen more than to native collagen, and exogenous PAI-1 only decreased tube length in endothelial cells on glycated collagen. (A) Plates
coated with native collagen, glycated collagen, or multimeric vitronectin were incubated with 1 μg/ml PAI-1. A solid-phase binding assay was used to detect bound PAI-1.
*p < 0.01 compared to native collagen. (B) 15,000 HUVEC were added to native or glycated collagen gels ± 12 IU/ml uPA or 1 ng/ml PAI-1. After 18 h, tubes were imaged by
phase contrast microscopy and tube length was analyzed with ImageJ. Samples were normalized to the control without uPA or PAI-1 on the respective substrate to best
compare uPA and PAI-1 effects. *p < 0.01 compared to samples without uPA or PAI-1 on the respective substrate. Each experiment was completed in triplicate and repeated
at least two times.
We next measured uPA and PAI-1 mRNA levels at the same time points to determine if changes in PAI-1 protein levels were related to PAI-1 production. Neither glycated collagen nor FGF-2 affected uPA mRNA at 2 h, which was consistent with Western blot data (Fig. 3A). Glycated collagen induced a small but significant increase in PAI-1 mRNA at 2 h, and FGF-2 decreased this effect (Fig. 3B). After 24 h, uPA mRNA was similar across all samples but FGF-2 decreased PAI-1 mRNA by 30% in both native and glycated collagen (Fig. 3C and D).

4.3. PAI-1 binding to glycated collagen

We and others previously showed that matrix protein denaturation and glycation can induce structural changes that alter cell and protein binding [30–33]. We therefore hypothesized that PAI-1 levels increased in cells on glycated collagen partially due to PAI-1 binding. Indeed, more than three times as much PAI-1 bound to glycated collagen as compared to native collagen in an equilibrium binding assay (Fig. 4). FGF-2 did not displace the PAI-1 bound to glycated collagen (data not shown). We then added exogenous uPA and PAI-1 to HUVEC cultured on native or glycated collagen gels. Whereas uPA increased capillary-like tube length on both substrates, PAI-1 only decreased tube length in cells on glycated collagen gels. These data suggest that PAI-1 binding to glycated collagen mediates its effect on angiogenic processes.

Glucose induced changes in the extracellular matrix, such as collagen glycation, decrease endothelial capillary-like tube formation in vitro [10]. We previously showed that glucose also increases endothelial FGF-2 release and matrix storage [11]. We now show that FGF-2 only partially restored plasminogen system activity in cells on glycated collagen. FGF-2 decreased PAI-1 levels in cells on glycated collagen. However, FGF-2 did not decrease PAI-1 binding to glycated collagen, which may localize PAI-1 to the extracellular matrix [30,31,34,35]. While PAI-1 binding and activity were previously shown to increase with glycated fibrin [32], we are the first to report that PAI-1 also binds to glycated collagen, and that this may modulate plasminogen system activity.

While these studies describe interesting new interactions among growth factors, glycated collagen, and the plasminogen system, they are not without limitations. We used HUVEC to relate our results to previous work in the literature and because they form capillary-like tubes in vitro. However, microvascular endothelial cells may be a better model for future work. In our experiments, FGF-2 did not affect uPA or PAI-1 mRNA. This was initially surprising, since others had previously shown that FGF-2 regulates both uPA and PAI-1 [24,25]. However, FGF-2 typically has less effect on the plasminogen system in human as compared to animal cells, and therefore continuous FGF-2 stimulation or inhibitor proteins may be required to enhance FGF-2 effects [14,24,36]. In fact, when we added fibrinogen to our FGF-2-stimulated samples, we did observe significant increases in uPA and PAI-1 mRNA. Finally, some of our observed effects on plasminogen system activity could relate to tissue plasminogen activator (tPA), rather than uPA. tPA is produced by endothelial cells, although it is primarily thought to generate plasmin for blood fibrinolysis [37]. We focused on uPA due to its importance in angiogenic functions; however the possibility of tPA effects cannot be excluded.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2015.09.001.

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