Introduction

More than 645,697 patients in the USA require hemodialysis for the purification of their blood due to end-stage renal disease [1]. The arteriovenous fistula (AVF) has become the first-line therapy for patients requiring hemodialysis vascular access. AVFs have been used for more than 50 years and are prone to venous stenosis formation [2]. Although the exact mechanism for venous stenosis is not yet clear, several major factors have been
hypothesized including hypoxia, shear stress, oxidative stress and inflammation [3, 4]. As a consequence, these factors lead to an accumulation of macrophages, fibroblasts and smooth-muscle cells in the vessel wall, that lead to stenosis formation [5]. Furthermore, hypoxia contributes to an increase in growth factors that induce fibroblast proliferation, migration and differentiation to myofibroblasts and result in fibrosis [6–8]. In addition to all of the above factors, hyperglycemia associated with diabetes exacerbates the formation of venous stenosis [9].

Our laboratory has used a hypoxic fibroblast cell culture system to understand the mechanisms responsible for venous stenosis formation. Previous results from our laboratory indicate that with a hypoxic stimulus, there is an increased expression of matrix metalloproteinase-2 (Mmp-2) accompanied by fibroblast-to-myofibroblast differentiation [10]. Moreover, inhibiting vascular endothelial growth factor-A (Vegf-A) gene expression in this model has been associated with a decrease in the differentiation of fibroblasts to myofibroblasts, which is accompanied by a reduction in the expression of Mmp-2 gene and MMP-2 protein [3]. In line with these results, the implantation of autologous blood outgrowth endothelial cells to the adventitia of the arterial venous grafts also decreases MMP-2 protein activity, accompanied by a decrease in venous stenosis formation [11]. However, under hypoxic stress, the effect of Mmp-2 gene knockdown in hyperglycemic cells and in euglycemic cells previously cultured in hyperglycemic milieu is unknown. This holds implications for patients with diabetes.

Experimental studies have shown that under hypoxic stress, fibroblasts will secrete VEGF-A, which is necessary for the coordination of multiple cellular functions [12–14]. Fibroblasts have a synthetic function (to secrete collagen I and IV for tissue remodeling to occur) and contain elaborate cytokines [MMPs, VEGF-A, platelet-derived growth factor (PDGF) and others] responsible for cell proliferation and migration [15, 16].

We used an in vitro fibroblast cell culture model using both hyperglycemia and hypoxia to simulate diabetic injury in hemodialysis vascular access. The goal of the study was to evaluate the loss of Mmp-2 function on fibroblasts acclimatized to hyperglycemia and then to euglycemia during hypoxia and normoxia. We investigated the gene expression of Vegf-A, Vegfr-1, Mmp-9 and tissue inhibitors of metalloproteinases (Timps). Functional analysis was performed to evaluate collagen I and IV synthesis and cellular migration and proliferation.

Materials and Methods

Monoclonal antibodies against β-actin (clone AC-15) and α-smooth-muscle actin (α-SMA) were purchased from Sigma-Aldrich, St. Louis, Mo., USA. Collagen I and IV were purchased from Rockland Immunochemicals Inc., Limerick, Pa., USA. DMEM and all other chemicals for cell culture were obtained from Invitrogen Inc., Waltham, Mass., USA, unless otherwise specified.

Cell Culture and Treatments

The experimental design is depicted in figure 1. AKR-2B murine fibroblast cell line was obtained from Dr. Edward Leof, Mayo Clinic, Rochester, Minn., USA. Cells were cultured in DMEM supplemented with glucose to a final concentration of 30 mM, plus 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. Cells were maintained at 37°C in a humidified CO₂ incubator with 5% CO₂ and 95% air [3].

Culture of AKR-2B Cells under Varying Glycemic Conditions

AKR-2B cells were preconditioned in hyperglycemic milieu (media containing 30 mM glucose). All experiments were performed with these preconditioned cells and considered as a hyperglycemic condition. A subculture of the hyperglycemia-preconditioned cells was grown in regular DMEM containing 5 mM glucose supplemented with 10% FBS, 1% penicillin and streptomycin, i.e. euglycemia-acclimatized cells.

Lentivirus-Mediated Mmp-2 Gene Silencing

To assess the efficacy of glycemic changes upon the invasiveness of fibroblasts and myofibroblasts, the expression of the con-

Fig. 1. Schematic representation of the experimental approach. Murine fibroblast (AKR-2B) cells were initially preconditioned to hyperglycemia (maintained in DMEM with 30 mM glucose). These cells were then acclimatized to euglycemia (subcultured in DMEM with 5 mM glucose). Both hyperglycemic and euglycemic cells were then silenced for the Mmp-2 gene using shRNA. The cells transduced with LV either silenced for Mmp-2 (LV) or scrambled shRNA (controls) were further subjected to functional experiments with varying indices of oxygen (normoxia and hypoxia).
Lentiviral-transduced AKR-2B cells were maintained under both euglycemic and hyperglycemic conditions, seeded at a density of 1 × 10^5 cells/cm² and allowed to grow to 50–60% confluency. Cells (under both euglycemic and hyperglycemic conditions) were serum-starved overnight with DMEM, supplemented with 10% FBS and then exposed to normoxic (21% O₂, 5% CO₂ and a balance of nitrogen) or hypoxic conditions (3% O₂, 5% CO₂ and a balance of nitrogen) at 37 °C.

AKR-2B cells grown under euglycemic or hyperglycemic conditions were subjected to hypoxic stress for 24 or 72 h, as were the respective normoxic controls. After the incubation period, cells were washed 3 times with prewarmed (37 °C) PBS and fixed in paraformaldehyde (4% PFA) in PBS for 30 min. After fixation, cells were washed twice in PBS and permeabilized with 0.1% Triton X-100 in PBS for 1 min. After washing 3 times with PBS, the cells were incubated in rhodamine-phalloidin (1:1,000 dilation, Sigma-Aldrich) in PBS for 30 min at room temperature. The cells were washed 5 times in PBS and mounted with mounting medium containing 50% methanol, 10% acetic acid and 0.5% Coomassie blue (DAPI; Vector Labs, Burlingame, Calif., USA). Images were acquired using a confocal microscope (excitation: 550 nm and emission: 580 nm) with x40 and x63 objectives as described previously [3, 4].

**Assessing Collagen Activity from Conditioned Media**

Zymography was performed on the conditioned media from cultured cells at completion of their respective treatments according to the protocol published by our group [10]. Protein (100 μg) was electrophoresed using 10% polyacrylamide gels containing 0.1% gelatin (Bio-Rad, Hercules, Calif., USA). After electrophoresis, the gels were incubated twice for 30 min in 10 volumes of 2.7% Triton X-100 renaturing buffer and once for 30 min in 10 volumes of development buffer [200 mM NaCl, 50 mM HEPES (pH 7.5), 5 mM CaCl₂, and 20 μM ZnCl₂]. The gels were then incubated at 37 °C in fresh development buffer overnight and subsequently stained in 40% methanol, 10% acetic acid and 0.5% Coomassie blue R-250 for 30 min, followed by destaining in the same solution without the dye. Bands were semiquantitated by reverse-image scanning densitometry (NIH ImageJ Software, NIH, Bethesda, Md., USA). An area of the gel image that was devoid of signal was assigned to be the background value. Each band was then analyzed for the density above the background. The results were calculated and expressed as relative densitometric units/unit area. Relative active protein was calculated by dividing the densitometric value in the active band by the total densitometric value of the active and proactive bands.

**Cell Proliferation Assay**

LV-transduced AKR-2B cells acclimatized to both euglycemic or hyperglycemic conditions were seeded in 24-well plates at a density of 5 × 10⁴ cells/ml and cultured for 48 h in their respective glycemic conditions. Cells were serum-starved (0.1% serum) overnight and subjected to hypoxic or normoxic conditions for 20 h and then 1 μCi of [³H]-thymidine was added to the media and incubated for another 4 h under their respective incubating conditions. After 4 h, cells were washed with ice-cold PBS, fixed with 100% cold methanol and collected for the measurement of trichloroacetic acid-precipitated radioactivity [18]. Experiments were repeated at least 3 times.

**Cell Migration Assay**

Chemotaxis was measured using a modified Boyden chamber migration assays (BD) with the use of 8-μm pore-size, polycarbonate transwell inserts coated with collagen as described previously [3, 4]. Briefly, Mmp-2-silenced AKR-2B cells were acclimatized to euglycemic or hyperglycemic conditions and suspended in serum-free medium and then seeded in the upper well (20,000 cells/well). The cells were then incubated for 4 h at 37 °C in a CO₂ incubator or hypoxia chamber, before being fixed with 4% PFA and stained with 0.2% crystal violet dissolved in 2% ethanol. Migration was quantified by counting the number of cells on the filter using bright-field optics with a Nikon Diaphot microscope (Nikon Instruments Inc., Melville, Ky., USA) equipped with a 16-square reticule (1 mm²). Cells that passed through the filter were quantified from random microscopic fields. Each assay was carried out in triplicate, with 6 replicates of each group per assay.

**Actin Dynamics in Fibroblasts**

AKR-2B cells maintained under euglycemic or hyperglycemic conditions were subjected to hypoxic stress for 24 or 72 h, as were the respective normoxic controls. After the incubation period, cells were washed twice in PBS and permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. The cells were washed 5 times in PBS and mounted with mounting medium containing 50% methanol, 10% acetic acid and 0.5% Coomassie blue (DAPI; Vector Labs, Burlingame, Calif.), Images were acquired using a confocal microscope (excitation: 550 nm and emission: 580 nm) with x40 and x63 objectives as described previously [3, 4].

**Hypoxia Induces Physiognomic and Biochemical Changes**

Lentiviral-transduced AKR-2B cells acclimatized to both euglycemic and hyperglycemic conditions were seeded in 24-well plates at a density of 1 × 10⁴ plaque-forming units/ml) carrying about 50–60% of confluency. One milliliter of LV solution (approx. 2 × 10⁷ plaque-forming units/ml) carrying Mmp-2 shRNA or control shRNA (Dharmacon, GE Healthcare, Lafayette, Colo., USA; accession Nos. NM_008610 and XM_006530751) and 5 ml of fresh medium were added to cells with 10 μg/ml polybrene and incubated for 16 h. The transduced cells were selected by subculturing in DMEM supplemented with 10% FBS and 1 μg/ml puromycin. All experiments were performed with the above puromycin-resistant cells.
cience (ECL) detection reagents (Rockford, Ill., USA) and exposed to X-OMAT AR films (Eastman Kodak, Rochester, N.Y., USA). The films were scanned on an EPSON scanner and the optical density (OD) of each band was determined using ImageJ software. The OD of bands in the control treatment was designated as 100 after being normalized to the respective loading controls (β-actin for α-SMA and Ponceau-S staining for collagen). All experiments were performed in triplicate.

Reverse Transcriptase Polymerase Chain Reaction
RNA from the cells was isolated as per manufacturer guidelines (Qiagen, Gaithersburg, Md., USA). First-strand complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand system (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer’s guidelines. cDNAs specific for the analyzed genes, Vegf-R1, Vegf-A, Mmp-2, Mmp-9, Timp-1 and Timp-2, were amplified using the primers obtained from SA Biosciences (Valencia, Calif., USA). PCR products were analyzed on 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. Bands were semi-quantitated by scanning densitometry (ImageJ). An area of the gel image that was devoid of signal was assigned to be the background value. Each band representing the gene of interest was then analyzed for the density above the background, and then normalized to ensure that there were no differences in the amount of loading of mRNA to the 18S gene.

Statistical Analysis
Data are expressed as mean ± SEM. Two-way ANOVA followed by Student’s t test with post hoc Bonferroni’s correction was performed. Significant differences between groups were indicated by p < 0.05, p < 0.01, p < 0.001 or p < 0.0001.

Results
Mmp-2 shRNA Suppresses the Expression of the Mmp-2 Gene and MMP-2 Protein in AKR-2B Cells
We determined Mmp-2 shRNA efficacy in fibroblasts by measuring the Mmp-2 gene and MMP-2 protein expression under experimental conditions. This included hyperglycemia and those cells acclimatized to euglycemia at 24 and 72 h of hypoxia and normoxia. Results from RT-PCR analysis revealed that Mmp-2 gene expression was significantly decreased in Mmp-2-silenced fibroblasts compared to scrambled controls under hypoxic conditions at 24 (p < 0.05) and 72 h (p < 0.01) of hypoxia and normoxia (fig. 2a). Similar results were observed in cells acclimatized to euglycemic conditions at 24 h of hypoxia (p < 0.05) and normoxia (p < 0.001) and at 72 h (p < 0.01) in cells silenced for Mmp-2 compared to controls (fig. 2a).

Next, we performed zymography to assess the effect of Mmp-2 gene knockdown on pro-MMP-2 activities in fibroblasts. Pro-MMP-2 activity was significantly decreased in hyperglycemic cells at 24 (p < 0.05) and 72 h (p < 0.05) of normoxia and hypoxia in Mmp-2-silenced fibroblasts compared to controls (fig. 2b). Under euglycemic conditions, Mmp-2-silenced fibroblasts had significantly decreased pro-MMP-2 activity, when cultured for 24 h under hypoxia and normoxia (p < 0.05) compared to respective controls. However, a reduction in pro-MMP-2 activity was observed only at 72 h of hypoxia (p < 0.01) in Mmp-2 knockdown fibroblasts acclimatized to euglycemic conditions compared to their respective controls (fig. 2B).

We next assessed active MMP-2 activity under similar conditions. Mmp-2-silenced fibroblasts had significantly reduced active MMP-2 activity in hyperglycemic cells at 24 (p < 0.05) and 72 h (p < 0.01) of normoxia and hypoxia compared to respective controls (fig. 2b). Similar results were observed in cells acclimatized to euglycemic conditions at 24 h of normoxia (p < 0.05) and hypoxia (p < 0.01) as well as at 72 h of normoxia (p < 0.05) in Mmp-2-silenced fibroblasts compared to respective controls (fig. 2b).

Loss of Mmp-2 Reverses the Hyperglycemia-Induced Vegf-A and Vegfr-1 Gene Regulation
We have previously shown that Vegf-A-silenced fibroblasts under hypoxic conditions have reduced MMP-2 activity compared to normoxic fibroblasts. Vegf-A and its cognate receptor Vegfr-1 are downstream intermediates of the hypoxic transcriptome and so Vegf-A and Vegfr-1 gene expression was assessed by RT-PCR. Our results demonstrate that under hyperglycemic conditions, there was a significant reduction in Vegf-A gene expression at 24 h of hypoxia (p < 0.05) in Mmp-2-silenced fibroblasts when compared to controls (fig. 3a). However, under euglycemic conditions, Vegf-A expression was significantly increased at 72 h of hypoxia (p < 0.05) in Mmp-2-silenced fibroblasts when compared to respective controls (fig. 3a).

Under both hyperglycemic and euglycemic conditions, Mmp-2-silenced fibroblasts had significantly decreased Vegfr-1 gene expression after being cultured for 24 h of normoxia (p < 0.05) compared to the controls (fig. 3b). These results conclusively demonstrate that the Mmp-2-silenced fibroblasts had a profound impact on the expression of Vegf-A and its cognate receptor Vegfr-1, irrespective of glycemic variance.

Mmp-2 Modulates the Expression of Genes Associated with Matrix Turnover under Varying Glycemic Conditions with Hypoxia and Normoxia
Mmp-2-silenced fibroblasts exposed to hyperglycemic conditions had a significant decrease in the average ex-
pression of the Mmp-9 gene at 24 h of normoxia (p < 0.05) and 72 h of hypoxia (p < 0.05) compared to respective controls (fig. 4a). However, under euglycemic conditions, the reduction in Mmp-9 gene expression was observed only at 24 h of normoxia (p < 0.05) in Mmp-2-silenced fibroblasts compared to respective controls.

Next, we determined the gene expression pattern of Timp-1, an inhibitor of Mmp-9. Under hyperglycemic conditions, Mmp-2-silenced fibroblasts had significantly reduced Timp-1 gene expression when cultured for 24 h (p < 0.05) of normoxia, but this increased significantly (p < 0.05) when the cells were exposed to hypoxia for 24 h compared to respective controls (fig. 4b). Under euglycemic conditions, Mmp-2-silenced fibroblasts had significantly increased Timp-1 expression at 24 (p < 0.01) and 72 h (p < 0.05) of hypoxia only compared to their respective controls.

Finally, we determined the pattern of gene expression of Timp-2, an inhibitor of Mmp-2. Under hyperglycemic conditions, Mmp-2-silenced fibroblasts significantly reduced the expression of the Timp-2 gene (p < 0.01) when exposed to 24 h of normoxia compared to the respective controls (fig. 4c).

Loss of Mmp-2 Reduces Hypoxia with Hyperglycemia-Mediated Fibroblast-to-Myofibroblast Differentiation

We have previously shown that Vegf-A-silenced fibroblasts have a significantly reduced α-SMA expression under hypoxic versus normoxic conditions [4]. We determined α-SMA expression by Western blot analysis in fibroblasts silenced for Mmp-2 and exposed to hyperglycemic and euglycemic conditions for 24 and 72 h with hypoxia or normoxia. Our results demonstrate that under hyperglycemic or euglycemic conditions, Mmp-2-silenced fibroblasts had significantly reduced α-SMA protein when exposed to 24 h of hypoxia or normoxia compared to the respective controls (fig. 5a).

We next determined the amount of actin stress fibers in Mmp-2-silenced fibroblasts cultured under hyperglycemic and euglycemic conditions and exposed to varying oxygen levels. Under hyperglycemic conditions, Mmp-2-silenced fibroblasts had significantly reduced actin stress fiber formation at 24 h of normoxia (fig. 5b; p < 0.01) and hypoxia (p < 0.001) and at 72 h (of both normoxia and hypoxia; p < 0.0001) when compared to controls. Under euglycemic conditions, there was a significant reduction in the average percentage change in actin stress fiber formation at 24 (p < 0.001) and 72 h of hypoxia only (p < 0.0001) compared to the controls.

Loss of Mmp-2 Alters Collagen Secretion

Collagen I and IV are substrates for MMP-2. We hypothesized that there would be a reduction in their activity in Mmp-2-silenced fibroblasts. We have demonstrated that Vegf-A shRNA transduced vessels have decreased expression of the Mmp-2 gene and MMP-2 protein expression, accompanied by a reduction in collagen I and IV staining as determined by Picrosirius red [19]. We determined collagen I and IV expression by performing Western blot analysis on the conditioned media. We observed that under hyperglycemic conditions, Mmp-2-silenced fibroblasts when compared to controls have a significant reduction in the average collagen I expression at 24 h of normoxia (fig. 6a; p < 0.05) and hypoxia (p < 0.01) as well as at 72 h of hypoxia only (p < 0.01). A reduction in collagen I expression was observed at 24 h of normoxia only (p < 0.05) under euglycemic conditions.

Loss of Mmp-2 Decreases Cell Migration under Hyperglycemia

Decreasing Mmp-2 expression can reduce the migratory potential of cells. We determined the migration of such cells using an in vitro invasion assay as assessed by the Boyden’s chamber. Our experiments demonstrated

![Fig. 2. Mmp-2 gene and MMP-2 protein expression in cells silenced for Mmp-2. AKR-2B cells acclimatized to hyperglycemic and euglycemic conditions were silenced for Mmp-2 (LV) or scrambled shRNA (controls, C). Mmp-2 gene expression was assessed by RT-PCR (a) and MMP-2 protein and enzyme activity by zymography (b) at 24 and 72 h of hypoxia and normoxia. Top: representative gel images. Bottom: densitometric quantification for respective treatment conditions shown in the upper panels. Each bar represents the mean ± SEM of 4–6 experiments per group. Two-way ANOVA followed by Student’s t test with post hoc Bonferroni’s correction was performed. * p < 0.05, ** p < 0.01 and * p < 0.001, significant difference from control value. (For figure see next page.)](http://jvascreslw/2015/52/334-346)
MMP-2-Dependent Hyperglycemia Induced Modulation of Fibroblast Physiognomy

DOI: 10.1159/000443886
**Fig. 3.** Gene expression of *Vegf-A* and *Vegfr-1* in hyperglycemic and euglycemic cells during hypoxia and normoxia. Expression of the *Vegf-A (a)* and *Vegfr-1 (b)* genes was assessed by RT-PCR in AKR-2B cells cultured in hyperglycemic and euglycemic media silenced for *Mmp-2 (LV)* or scrambled shRNA (controls, C) subjected to hypoxia or normoxia for 24 and 72 h. Top: representative agarose gel images of gene expression. Bottom: densitometric analysis of gene expression under the respective glycemic conditions. Each bar represents the mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student’s t test with post hoc Bonferroni’s correction was performed. *p < 0.05, significant difference from control value.

**Fig. 4.** Expression of the *Mmp-9 (a)*, *Timp-1 (b)* and *Timp-2 (c)* genes was assessed by RT-PCR in AKR-2B cells cultured in hyperglycemic and euglycemic media silenced for *Mmp-2 (LV)* or scrambled shRNA (controls, C) subjected to hypoxia or normoxia for 24 and 72 h. Top: representative agarose gel images of gene expression. Bottom: representative densitometric analysis of gene expression under the respective glycemic conditions. Each bar represents the mean + SEM of 4–6 experiments per group. Two-way ANOVA followed by Student’s t test with post hoc Bonferroni’s correction was performed. *p < 0.05 and **p < 0.01, significant difference from control value.

(For figure see next page.)
Fig. 5. Western blots of α-SMA and phalloidin staining to assess stress fiber formation. Conversion of fibroblasts to myofibroblasts was assessed by measuring α-SMA protein using Western blot (a) and immunofluorescence staining for phalloidin (b) in cells silenced for Mmp-2 (LV) or scrambled shRNA (controls, C) subjected to hyperglycemic or euglycemic conditions under hypoxia or normoxia for 24 and 72 h. a Representative images of α-SMA (top) and densitometric quantification (bottom). b Representative images of phalloidin staining (top) with image quantification (bottom) of the respective glycemic conditions. Each bar represents mean ± SEM of 4–6 experiments per group. Two-way ANOVA followed by Student’s t test with post hoc Bonferroni’s correction was performed. * p < 0.05, ** p < 0.01, * * p < 0.001 and ## p < 0.0001, significant difference from control value.
Fig. 6. Collagen I and IV expression in hyperglycemic and euglycemic cells during hypoxia and normoxia. Expression of collagen I (a) and IV (b) was assessed using Western blot performed on the conditioned media from cells silenced for *Mmp-2* (LV) or scrambled shRNA (controls, C) and subjected to hyperglycemic or euglycemic conditions during hypoxia or normoxia for 24 and 72 h.

Top: representative images of Western blots. Bottom: densitometric analysis. Each bar represents mean ± SEM of 4–6 experiments per group. Two-way ANOVA followed by Student’s t test with post hoc Bonferroni’s correction was performed. *p < 0.05 and **p < 0.01, significant difference from control value.
that in Mmp-2-silenced fibroblasts under hyperglycemic conditions had a significant reduction in the migratory potential (fig. 7a; p < 0.001) when exposed to hypoxia and normoxia compared to controls. Under euglycemic conditions, no significant difference was observed between the controls and cells silenced for Mmp-2.

Loss of Mmp-2 Alters Cell Proliferation with Varying Concentrations of Oxygen and Glucose

Proliferation was determined using a thymidine incorporation assay. Our results demonstrated that Mmp-2-silenced fibroblasts conditioned to hyperglycemic milieu under hypoxic conditions (fig. 7b; p < 0.001) or to euglycemic milieu under both hypoxic (p < 0.001) and normoxic stress (p < 0.0001) underwent a significant increase in the percentage change in the proliferative potential of cells.

Discussion

Hemodialysis vascular access failure caused by venous stenosis formation is a significant clinical problem for patients with end-stage renal disease and it is associated with worse clinical outcomes in those patients with diabetes [9]. A majority of patients on hemodialysis often have an underlying history of diabetes [9], and a recent study demonstrated that fistula patency was associated with optimal glycemic control [21]. Developing novel therapies to aid in reducing venous stenosis formation is important for improving patient outcomes.

MMP-2, a constitutive matrix metalloproteinase plays a key role in the invasive potential of cells including fibroblasts in vascular stenosis [4, 22]. Pharmacological inhibition of MMPs has been used as a therapeutic strategy for cancer; however, a major problem is the selectivity issues associated with these inhibitors [23]. Furthermore, an antisense approach has been used to decipher the role of MMP-2 in cancer progression [22]. To the best of our knowledge, the role of MMP-2 in vascular stenosis has not been elucidated in diabetic milieu. This prompted us to investigate the simultaneous role of hyperglycemia and hypoxia on the physiognomy of the adventitial/medial fibroblasts. Previous studies from our laboratory suggest that Mmp-2 plays major role in hypoxia-induced fibroblast-to-myofibroblast differentiation [6]. In this study, we used an in vitro fibroblast cell culture model that employed both hyperglycemia and hypoxia to partly simulate epithelial-to-mesenchymal differentiation, focusing on fibroblast function and biology.
The turnover of the extracellular matrix facilitates the migration of tumor cells causing tumor metastasis [24] and of adventitial fibroblasts to the lumen of blood vessels, thereby significantly contributing to the onset of vascular stenosis [4]. Copious amounts of collagen I and IV were produced by the fibroblasts acclimatized to euglycemia compared to hyperglycemic fibroblasts in this study. In line with these results, glucose-induced MMP-2 and MMP-9 activity with a decrease in collagen synthesis by fibroblasts and endothelial cells has been described [25–27]. Surprisingly, the Mmp-2 gene did not have a statutory effect either under normoxic or hypoxic conditions, especially at the 72-hour time point. In line with these results, the loss of Mmp-2 decreased fibroblast migration under hyperglycemic conditions, while it had no effect on the migratory behavior of fibroblasts acclimatized to the euglycemic environment. Another possibility is that Mmp-9 and Mmp-2 activity, which was unaltered, might have increased the hypoxic stress-induced migratory behavior of those fibroblasts acclimatized to the euglycemic environment. A similar trend was observed with other physiologically relevant end points such as actin stress fibers, α-SMA, Mmp-9 and Timp-1. Together, these results indicate that fibroblasts that have been silenced for Mmp-2 gene expression and acclimatized to euglycemia from hyperglycemic conditions behave differently under hypoxic stress when compared to hyperglycemic cells.

Although hypoxia induces Vegf-A expression [28], this effect was observed only in Mmp-2-silenced cells acclimatized to euglycemic conditions when subjected to hypoxia for 72 h. In line with these results, high glucose disrupts the binding of HIF-1α to p300, a cofactor for HIF-1α via glycosylation leading to impaired HIF-1α-mediated transactivation of genes including Vegf-A [29]. Another possibility is that hyperglycemia can impair the hypoxia-dependent HIF-1α stability of proteosomal degradation [30–33]. It is possible that a similar interaction might have occurred, not only in the hyperglycemic cells, but also in the cells acclimatized to the euglycemic conditions.

Taken collectively, our studies indicate that glycemia plays an important role in modulating the behavior of fibroblasts. Mmp-2 gene silencing affected the physiognomy and behavior of the fibroblasts under hyperglycemic conditions. However, acclimatization to euglycemic conditions demonstrated results contrary to our expectations. These results indicate the statutory effect of the Mmp-2 silencing did not impact the physiognomy or the physiology of cells acclimatization to euglycemic conditions, particularly at the 72-hour time point. Moreover, the cellular response, such as hypoxia-mediated Vegf-A expression and the robust reappearance of the cortical stress fibers in fibroblasts acclimatized to euglycemia, is an indication of the 'metabolic memory' of these cells. Such results are indicative of the fact that tight management of the glycemic indices alone will not significantly alter or delay venous stenosis formation associated with hemodialysis vascular access failure. Therefore, these findings have implications for diabetic patients with hemodialysis vascular access.

We investigated fibroblast response after the transition from hyperglycemia to a euglycemic environment. One limitation of our study is the lack of negative control cells that were not previously cultured in a hyperglycemic environment. In addition, advanced glycation end products (AGE) levels in fibroblasts cultured under varying glycemic conditions might strengthen the concept of metabolic memory.

In summary, we conclude that Mmp-2 silenced fibroblasts under hyperglycemic conditions during hypoxic stimulus have an impaired response to Vegf-A/Mmp-9 expression. This results in a decrease in collagen I and IV secretion with a decrease in cell migration. These findings demonstrate a rationale for novel site-specific anti-MMP therapies aimed at maintaining the AVF patency. Moreover, maintenance of glycemic indices could help in significantly improving the performance of the AVF in diabetic patients. Further studies are required to validate our in vitro observations.

Acknowledgments

This work was funded by a RO1: HL098967 (S.M.) from the National Heart, Lung and Blood Institute.

Disclosure Statement

The authors have no conflict of interest to disclose.

References

20 Janardhanan/Kilari/Leof/Misra
558