Glycated Collagen Stimulates Differentiation of Gingival Myofibroblasts

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Background: Glucose-derived metabolites may alter the structure and biologic properties of important proteins in periodontium, such as collagens. As a consequence, it is possible that collagen-binding cells may change their phenotypic traits. Although the glucose-derived product methylglyoxal (MGO) has been detected in periodontal lesions, the precise effect of collagen glycation on gingival connective tissue biology is not fully understood. The present study evaluates whether collagen glycation by MGO may affect phenotypic properties and remodeling capacity of human gingival fibroblasts (HGFs).

Methods: Primary cultures of HGFs were grown on Type I collagen matrices previously treated with MGO. Cell cultures were tested for cell viability, apoptosis, α-smooth muscle actin (SMA), fibronectin (FN) production, and collagen remodeling. Mechanical properties and morphology of MGO-treated collagen gels were evaluated using rheometry and atomic force microscopy. Statistical analysis was performed by Kruskal-Wallis and Mann-Whitney *U* tests.

Results: MGO-treated collagen did not affect cell viability or apoptosis. In addition, MGO did not induce significant changes in morphology or mechanical properties of the collagen matrix. However, MGO-treated collagen stimulated an increase in the myofibroblast marker α-SMA, production and assembly of FN, and contraction of collagen matrices. Moreover, use of a triple-helical peptide that reconstitutes the collagen-binding domain for integrins GFOGER reverted the assembly of FN induced by MGO-treated collagen.

Conclusions: The present study shows that collagen glycation by MGO stimulates differentiation of myofibroblasts and production and assembly of FN. These responses may alter the homeostatic balance and wound-healing response of gingival connective tissues affected by diabetes mellitus or aging. *J Periodontol 2017;88:926-935.*

KEY WORDS

Aging; collagen; connective tissue; diabetes mellitus; fibroblasts; fibrosis.

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Methylglyoxal (MGO) corresponds to a product derived from glucose metabolism with potential cytotoxic effects on cells.[1] It has been proposed that MGO may contribute to degenerative changes associated with diabetes mellitus (DM) or aging in the peritoneum, myocardium, ocular lens, and skin.[2-5] Although MGO has been identified in the gingival crevicular fluid (GCF) of inflammatory periodontal lesions,[6] the precise effect of MGO on behavior of gingival connective tissues is not completely understood. Fibroblasts actively interact with collagen molecules in gingival tissues through integrin receptors, and modifications of this interaction are critically important in connective tissue homeostasis.[7] The biologic activity of MGO has been explained by its high reactivity with basic amino acids such as arginine and lysine.[8] These reactions are characterized by formation of cross-linked proteins generating so-called advanced glycation end products (AGEs).[8] The sequence GFOGER is the principal collagen-binding domain recognized by integrins in Type I collagen.[9] More specific studies have identified that MGO modifies the arginine residue present in this sequence, altering the interaction between glycated collagen and gingival fibroblasts delaying cell migration and collagen remodeling.[10,11] Moreover, soluble MGO induces apoptosis in gingival fibroblasts, reducing the number of cells available for wound healing.[12] A recent study has indicated

that MGO and other glucose-derived products may induce changes in the extracellular matrix (ECM) of diabetic skin.[13] Importantly, collagen-associated AGEs were able to predict long-term complications in patients with type 1 DM, emphasizing the role of collagen-associated changes in tissue dysfunction.[13]

Myofibroblasts are characterized by the presence of cytoskeletal protein α-smooth muscle actin (SMA)[14] and by reinforced cell contacts with ECM.[15] Differentiation of myofibroblasts is mainly driven by the cytokine transforming growth factor (TGF)-β1 and by increased levels of physical tension sensed by cells from remodeling ECM.[16,17] Culture of cardiac fibroblasts on MGO-treated collagen stimulates differentiation of myofibroblasts that probably contribute to fibrosis in the diabetic myocardium.[3,18] Myofibroblasts actively secrete and remodel FN networks during tissue healing.[19] Moreover, an abundant and disorganized FN matrix is usually observed during tissue fibrosis.[20] FN assembly is a particularly important event as it precedes the deposition of collagen molecules during maturation of ECM.[21] The present study assesses whether MGO-modified collagen may affect critical responses involved in connective tissue homeostasis, such as differentiation of myofibroblasts, remodeling of collagen gels, and production and organization of FN.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of human gingival fibroblasts (HGFs) were obtained from healthy gingival tissue surrounding bicuspids obtained from non-smoking, systemically healthy individuals at the Clinical Dental Facility, School of Dentistry, Pontificia Universidad Católica de Chile, Santiago, Chile. Periodontal examination demonstrated sites with probing depth <4 mm, no attachment loss, and no bleeding on probing. Tissues were obtained from six donors (two males and four females, aged 11 to 13 years; mean age: 12 years). This study protocol was approved by the institutional review board for human subjects from the Pontificia Universidad Católica de Chile (protocol no. 12-205). All donors agreed to participate in the study and their parents or guardians provided written informed consent. Individuals donating tissue samples were enrolled between April 2014 and December 2015. Cells were cultured in D-minimal essential medium‖ containing 10% fetal bovine serum¶ and penicillin streptomycin# at 37°C in a 5% CO_2 atmosphere. Experiments were performed at early cell passages (4 to 8) and were validated on at least three independent occasions using cells from at least three different donors.

Treatment of Collagen With MGO

Collagen glycation was performed following a previously described protocol.[10] Briefly, cell culture plates were treated with 2 mL Type I collagen solution** (50 μg/mL) and neutralized with 200 μL 0.1 N NaOH†† for 2 hours at 37°C, washed with phosphate-buffered saline (PBS), and incubated with MGO‡‡ (1 to 10 mM) for 48 hours at 4°C. MGO concentrations used in these experiments were selected considering previous studies that have identified MGO levels ≤23 mM in GCF of patients with periodontitis.[6] Aminoguanidine (AMG)§§ was used as a control to inhibit glycation.[3] For specific experiments, collagen or MGO-treated collagen was incubated with 10 μg/mL of a synthetic triple-helical peptide that reconstitutes the collagen-binding domain recognized by integrins GFOGER or a control peptide GPP-10.‖‖ To identify collagen changes by MGO, collagen samples were lysed in radioimmunoprecipitation assay buffer supplemented with 10% sodium dodecyl sulfate (SDS) at 70°C. Samples were fractioned using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8%) under reduced conditions followed by silver staining, as previously described.[22] In addition, collagen samples were lysed as described above, fractioned through SDS-PAGE, transferred to polyvinylidene difluoride transfer membrane.¶¶ Membranes were exposed to primary antibodies (Type I collagen## or MGO-modified proteins***) and secondary antibodies coupled to horseradish peroxidase††† and developed using a chemoluminescent reagent.‡‡‡

Western Blot

α-SMA and FN protein levels were detected through Western blot, as described above. To detect FN protein levels, a two-phase cell lysis procedure that allows detection of non-assembled deoxycholate (DOC)-soluble or assembled DOC-insoluble cell lysate was performed, as previously described.[23]

Cell Viability and Apoptosis Assays

HGF were seeded over collagen or MGO-treated collagen in 96-well plates (5,000 cells per well). Cells were maintained in serum-free cell culture media and cell viability was evaluated after 48 or 72 hours through a cell viability assay that evaluates mitochondrial metabolic activity.§§§ For evaluation of apoptosis, cells were seeded on collagen-coated coverslips, treated or not with MGO, and apoptotic cell death was evaluated

through terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay using a commercial kit.[‖‖‖] Staurosporine[¶¶¶] was used as a positive control of apoptosis.

Immunofluorescence

Cells were seeded over collagen-coated coverslips previously treated or not with MGO. Cells were fixed with 4% paraformaldehyde, permeabilized, blocked with bovine serum albumin, and incubated with antibodies for α-SMA,[###] FN, or anti-MGO-modified proteins.[****] The antigen–antibody complex was washed and incubated with appropriate secondary antibodies.[††††] Nuclei and actin cytoskeleton were stained with 4′,6-diamidino-2-phenylindole (DAPI) and phalloidin,[‡‡‡‡] respectively. Cells were observed and photographed using an epifluorescence microscope.[§§§§]

Collagen Gel Contraction Assays

Type I collagen solution[‖‖‖‖] (1 mg/mL) was neutralized with NaOH and incubated for 1 hour at 37°C. Collagen gels were treated with PBS or MGO (1 to 10 mM) for 48 hours at 37°C, and 400,000 cells were seeded over the gels. Both free-floating and attached collagen gel assays were performed. Gels were finally fixed (4% paraformaldehyde), followed by immunocytochemical detection of FN and DAPI. Quantification of gel contraction was estimated by πR^2.

Rheometric Measurement of Young's Modulus

Measurements of physical properties of collagen gels were obtained through a rheometer consisting of two parallel plates making a 1-mm gap and subjecting the sample to simple shear. The lower plate was kept fixed, whereas an alternative linear motion was imposed on the upper plate (10×10 mm^2) through a micropositioning system.[¶¶¶¶] Force cell equipment[####] sensed the force as a function of deformation. Samples were cut to fit exactly in the gap, preventing lateral excess. Parallel grooves on active surfaces, ≈0.05 mm deep and 0.3 mm apart, oriented perpendicularly to the shear direction prevented sample sliding. Average shear modulus (G) was determined for collagen gels from the slope of the stress–strain curve, and the resulting value was averaged for 20 cycles.

Atomic Force Microscopy (AFM)

To obtain a thin layer of collagen fibrils suitable for AFM, 50 μL collagen (5 μg/mL) solution was incubated over highly oriented pyrolytic graphite (HOPG) for 20 minutes. MGO (5 mM) was deposited over HOPG collagen at 37°C for 48 hours. After each step, excess fluid was eliminated through nitrogen flow and samples were washed with ultrapure water[*****] and dried under nitrogen. All images were obtained in tapping mode in air using cantilevers of 3.5 N/m spring constant and reflective coating. Images were acquired using high-resolution imaging

equipment.[†††††] Mechanical characterization of collagen fibrils, treated or not with MGO, was performed through nanoindentation in which approaching curves allow sample stiffness measurements and retraction curves permit adhesion assessment through the snap-off force, following previously published protocols (see supplementary Appendix 1 in online *Journal of Periodontology*).[24,25] All these measurements were performed on at least three independent occasions.

Enzyme-Linked Immunosorbent Assay (ELISA)

Protein levels for FN were determined using a commercially available ELISA kit.[‡‡‡‡‡]

Statistical Analyses

Data were analyzed using Kruskal-Wallis, Mann-Whitney U, or κ statistics tests using commercial software.[§§§§§] $P < 0.05$ was considered significant.

RESULTS

Effects of MGO on Collagen Glycation

Type I collagen samples were treated with different concentrations of MGO, as described in Materials and Methods. After electrophoretic separation of collagen samples and gel staining, a shift was observed in the migration of bands corresponding to Type I collagen (Fig. 1A). In addition, the MGO scavenger AMG inhibited this response. Generation of AGEs by MGO on collagen was visualized using immunofluorescence. To do so, collagen samples were treated with 1 mM MGO or 1 mM MGO plus 20 mM AMG for 48 hours. As shown in Figure 1B, MGO induced increase in the immunofluorescence signal compared with PBS-treated collagen or MGO plus AMG. To complement these experiments, the relative proportion of Type I collagen and MGO-modified proteins was analyzed using Western blot of PBS or MGO-treated collagen samples (1 and 5 mM). As shown in Figure 1C, treatment of collagen with MGO caused reduction in detection of Type I collagen bands and delay in their electrophoretic migration. In addition, treatment of collagen with MGO was associated with increase in bands corresponding to MGO-modified proteins and delay in their migration. These results strongly suggest that MGO was able to glycate the collagen matrix under study.

Effects of MGO-Modified Collagen on Cell Viability and Apoptosis

To analyze whether MGO-treated collagen induced change in cell viability, serum-starved HGFs were cultured on Type I collagen, treated or not with MGO. After 72 hours, no significant changes in cell viability were observed after culturing cells over MGO-treated collagen (see supplementary Fig. 1A in online *Journal of Periodontology*). The TUNEL staining method was used to analyze whether apoptotic events increased in cells cultured over MGO-treated collagen. Supplementary Figure 1B in online *Journal of Periodontology* shows representative images of these observations, where no changes were observed in DNA fragmentation associated with apoptotic cell death. This observation was verified after quantifying these results as shown in supplementary Figure 1C in online *Journal of Periodontology*.

Effects of MGO on Mechanical and Morphologic Properties of Collagen

Changes in collagen were investigated through assessment of the shear modulus in PBS (control) and MGO-treated collagen samples. Averages of shear modulus for PBS and MGO-treated collagen samples achieved a similar result ($G_{control}$ = 82 Pa and $G_{MGO\text{-}treated}$ = 80 Pa, respectively). The Young's modulus (E) was calculated using $E = 2G(1 + \upsilon)$, and by assuming that the gel is incompressible; that is, Poisson ratio (υ) is close to $\upsilon = 0.5$. Thus, the corresponding Young modulus did not show significant differences between PBS and MGO-treated collagen ($E_{control}$ = 246 Pa and $E_{MGO\text{-}treated}$ = 240 Pa). Nanoindentation using AFM allowed direct evaluation of both the local Young modulus and the adhesion of collagen fibrils.[24,25] Using this approach, the typical force curve displays an elastic regimen (Fig. 2A, blue) that is mainly characterized by the Young modulus of fibrils. At early stages of sample–tip contact (Fig. 2A), the force is attractive due to presence of adhesion, which is also clearly visible in the retracting curve (Fig. 2A, red). The local Young modulus was first obtained in non-glycated collagen through the fit of the data with a suitable functional form.[24] The best fit of a representative set of approaching curves (Fig. 2B) leads to Young modulus of E^F = 7.5 GPa (standard deviation, σ = 1.3 GPa) and adhesion work of w_{adh} = 3.0 J/m^2 (σ = 0.7 J/m^2). For MGO-treated fibrils, the Young's modulus was E^F_{MGO} = 3.6 GPa (σ = 1.8 GPa), about half that of non-treated fibrils. Conversely, the adhesion work significantly increased under action of MGO (w_{adhMGO} = 5.1 J/m^2) (σ = 0.8 J/m^2). Therefore, using nanoindentation with AFM, treatment of collagen with MGO resulted in increase in adhesive properties of the collagen fibrils and reduction of their elastic modulus. At larger forces, the cantilever tip penetrates fibrils and the force is rather a linear function of penetration (Fig. 2A). To characterize irreversible deformation of collagen fibrils under action of the indenter at high penetration, slope (K_P = $\Delta F/\Delta D$) of the indentation curve was measured (Fig. 2A). K_P informs about stiffness (Newton meter) and provides qualitative information of the plastic limit of fibrils. Probability distribution of K_P indicated that MGO-treated collagen was softer than PBS-treated collagen (Fig. 2C). In addition, AFM images of control and MGO-treated collagen (Figs. 2D and 2E) did not demonstrate conformational changes in collagen fibrils. This was also verified by direct measurements of the typical periodicity of collagen fibrils (gap size plus overlap segment), obtaining average periodicity of 69 nm, with σ = 6 nm in both cases. Tip–collagen adhesion increased in MGO-treated collagen. However, MGO effect was not homogeneous along fibrils (Figs. 2F and 2G), as reflected by periodicity of $\approx$70 nm along fibrils visible in the adhesion map of MGO-treated fibrils; larger adhesion occurred at gaps compared with overlaps. To
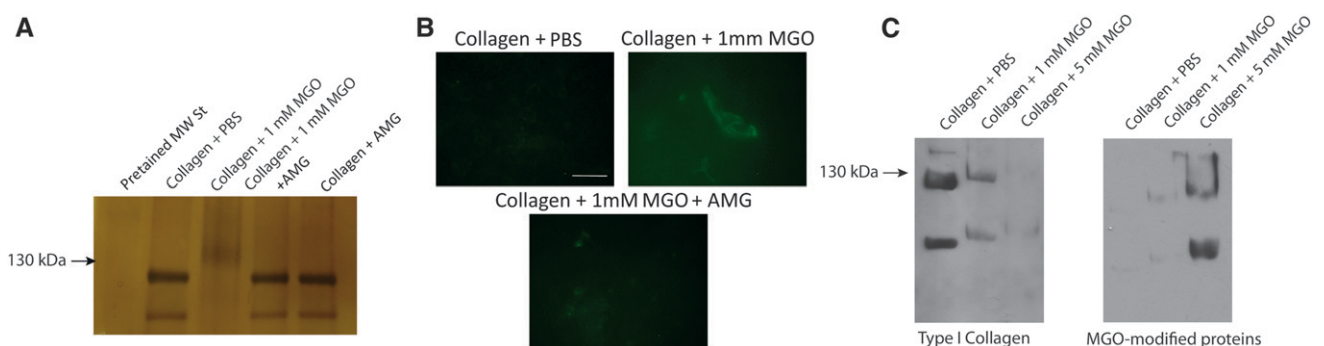

Figure 1.
Effects of MGO on collagen glycation. **A)** *Aliquots of collagen treated with PBS, 1 mM MGO, and/or 20 mM AMG for 48 hours. Image shows a silver stained gel of collagen samples. Molecular weight standard (MW St).* **B)** *Coverslips coated with collagen and subsequently treated or not with 1 mM MGO and/or AMG. Bar = 10 μm. Original magnification ×20.* **C)** *Aliquots of collagen treated with PBS or MGO (1 or 5 mM) for 48 hours.*
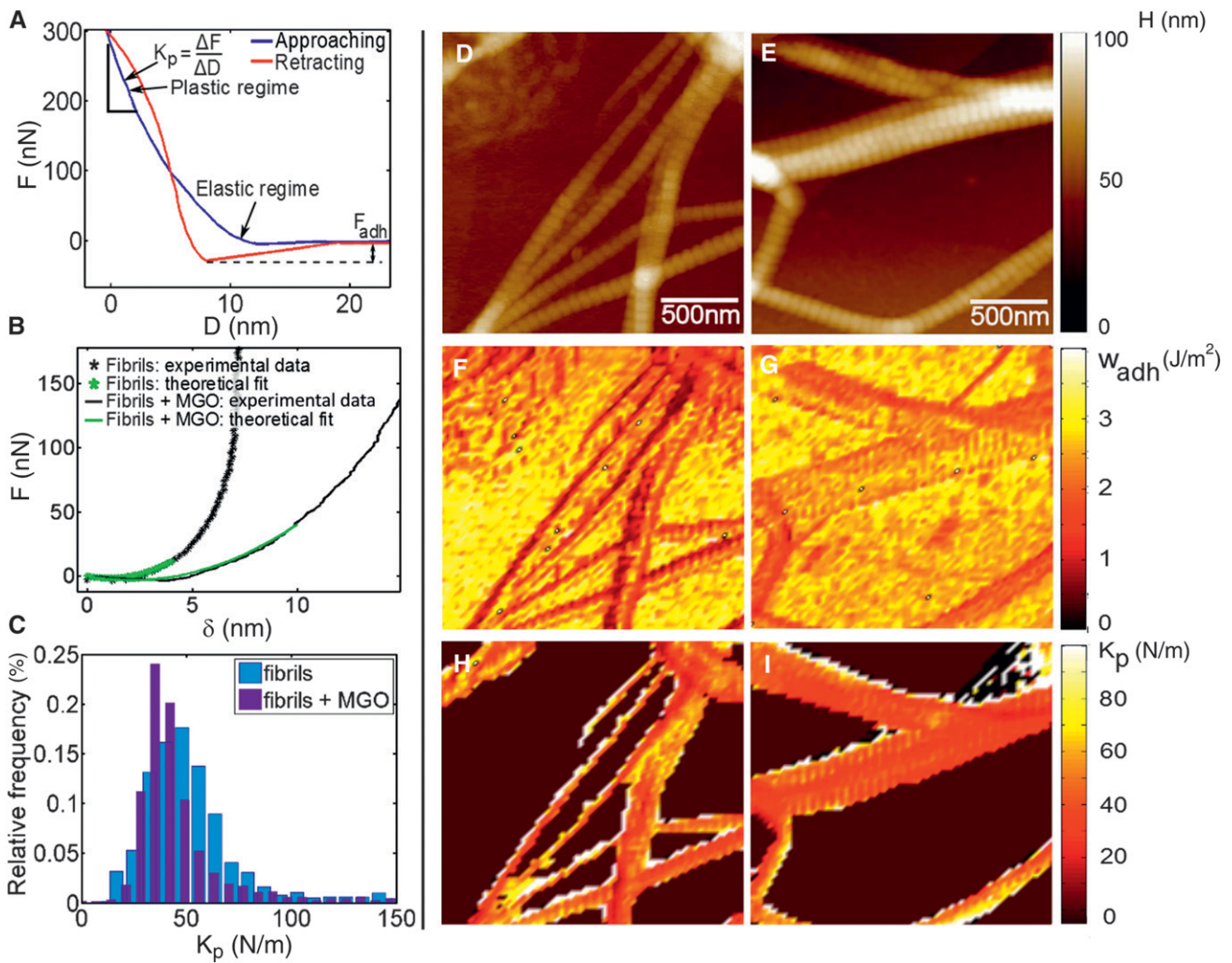
Figure 2.

*Mechanical and morphologic changes induced by MGO on collagen fibrils. **A)** Typical force curves in approaching (blue) and retracting (red). Whereas approaching is featured by elastic and plastic regimens, retraction presents significant adhesion characterized by F_{adh}. **B)** Typical force deformation curves in the elastic regimen for fibrils with and without MGO. Green lines represent the fits for the Young modulus. **C)** Statistical distribution of the plastic deformation parameter K_P, for fibrils with and without MGO. AFM maps: non-treated collagen fibrils (middle panels) and MGO-treated fibrils (right panels). **D** and **E)** Amplitude images obtained in tapping mode. **F** and **G)** Maps of adhesion work, w_{adh}. **H** and **I)** Maps of stiffness, K_P.*

characterize this feature, maps of stiffness K_P were recorded for fibrils before and after MGO treatment (Figs. 2H and 2I), revealing periodicity of ≈70 nm along MGO-treated fibrils and confirming MGO selectivity due to presence of gaps and overlapping regions.

MGO-Treated Collagen Stimulates Myofibroblastic Differentiation

To identify whether MGO-treated collagen was able to modulate differentiation of myofibroblasts, HGFs were seeded over collagen or MGO-treated collagen (1 mM) and α-SMA protein levels were evaluated in cell lysate after 72 hours. As a positive control, cells were also stimulated with 5 ng/mL TGF-β1. As shown in Figure 3A, cells cultured over MGO-treated collagen were characterized by moderate increase in α-SMA

protein levels (Fig. 3A). In addition, a more robust response on α-SMA protein levels was observed when cells were stimulated with TGF-β1, both in cells cultured over collagen or MGO-treated collagen (Fig. 3A). After quantifying the digital bands obtained from Western blots, significant increase in α-SMA protein levels was observed in cells cultured over MGO-treated collagen compared with non-treated collagen (Fig. 3B). α-SMA positive cells were evaluated using immunofluorescence (Fig. 3C). Although TGF-β1 induced the most potent increase in myofibroblastic differentiation, quantification of α-SMA positive cells showed significant increase in proportion of myofibroblasts in cells cultured over MGO-treated collagen compared with non-treated collagen (Fig. 3D).
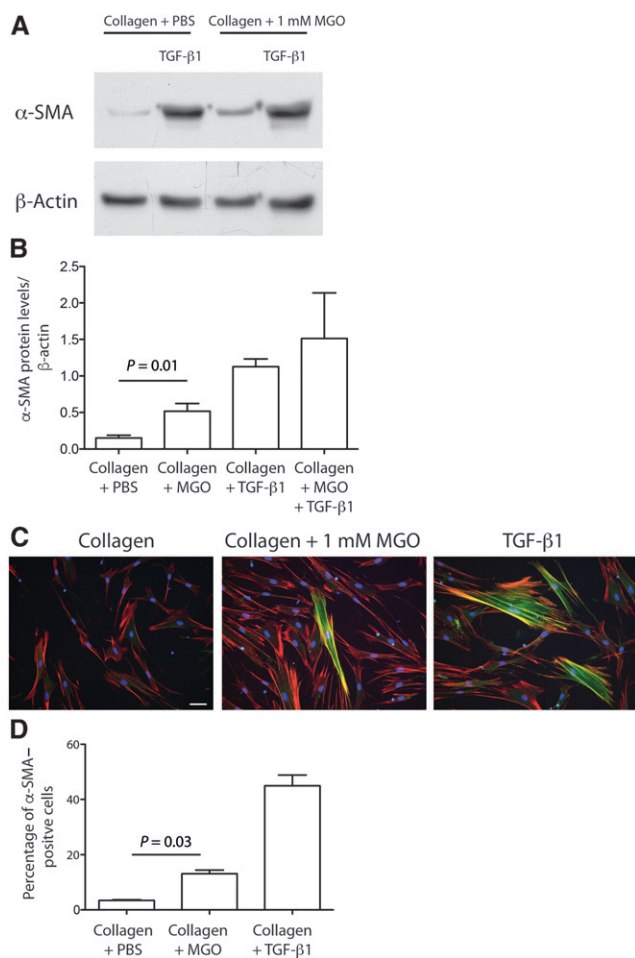
MGO-Treated Collagen Stimulates FN Protein Levels and Assembly

HGFs were seeded over coverslips previously treated with collagen or MGO-treated collagen, and FN distribution was evaluated through immunofluorescence. As shown in Figure 4A, MGO-treated collagen was associated with increase in the immunofluorescence signal for FN and appearance of a complex network for this protein as well. Therefore, a two-step cell lysis procedure was performed using detergent DOC to differentiate between loosely organized FN from the assembled condition of this protein. As shown in Figure 4B, Western blotting of the cell ly-

sates of DOC-soluble fractions demonstrated modest increase in FN levels of cells grown over MGO-treated collagen compared with non-treated collagen. However, increase in FN protein levels was detected in the DOC-insoluble fraction of cells grown over MGO-treated collagen. Quantification of these results showed a significant rise in total and DOC-insoluble FN protein levels when HGFs were cultured over MGO-treated collagen (Fig. 4C). ELISA was used to determine protein levels for FN in cell culture media and non-significant increase in soluble FN was observed when cells were cultured over collagen previously treated with 5 or 10 mM MGO (Fig. 4D). Because the major effect of MGO on collagen is probably due to glycation of arginine residues present in the GFOGER sequence recognized by collagen-binding integrins,[10,26] a GFOGER peptide was used to revert the increase in assembled FN induced by MGO-treated collagen. Serum-starved HGFs were seeded on collagen or MGO-treated collagen in the presence of the GFOGER peptide or the control peptide GPP-10. As shown in Figure 4E, addition of the GFOGER peptide reduced the amount of assembled (DOC-insoluble) FN induced by MGO-treated collagen. Meanwhile, no effect was found for the control peptide GPP-10. In addition, no effect was found for the GFOGER peptide on the DOC-soluble fraction. Quantification of these results demonstrated a significant decrease in amount of assembled FN in the presence of the GFOGER peptide (Fig. 4F).

MGO Stimulates Collagen Gel Remodeling

Using cells grown over three-dimensional collagen gels treated or not with MGO, increase in the signal for FN was observed in gingival fibroblasts cultured over collagen gels treated with 5 mM MGO resembling a mesh-like structure (Fig. 5A). These images were quantified showing significant increase in FN protein levels in cells cultured over MGO-treated collagen gels (Fig. 5B). Effect of MGO on collagen remodeling was also evaluated. Increase in collagen gel contraction was detected in cells grown over restrained collagen gels previously treated with MGO compared with non-treated collagen (Fig. 5C). Similar experiments using free-floating collagen gels did not demonstrate this response (data not shown). MGO scavenger AMG was used to confirm the role of collagen glycation on collagen gel contraction (Fig. 5D).

DISCUSSION

The present study explores whether collagen glycated by the glucose-derived product MGO may modify behavior of fibroblasts that interact with this matrix protein. Primary cultures of HGFs showed that these cells remain viable when cultured over MGO-treated collagen and develop a myofibroblastic differentiation program characterized by expression of
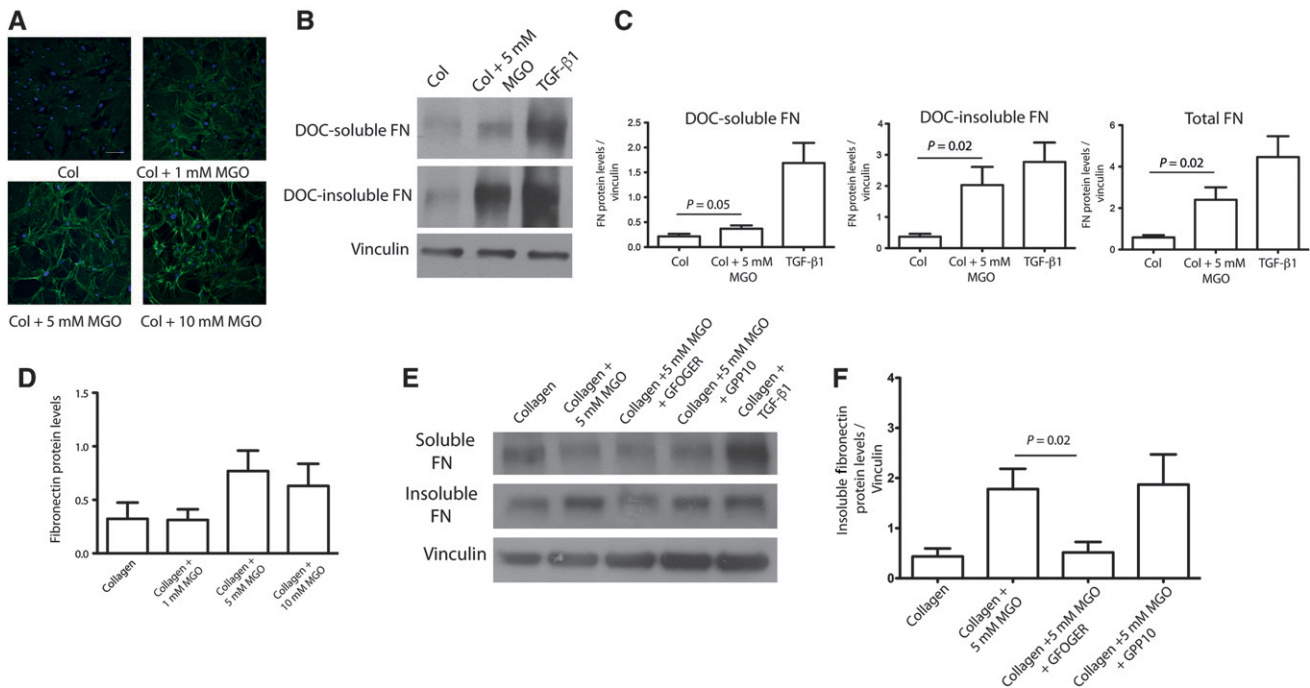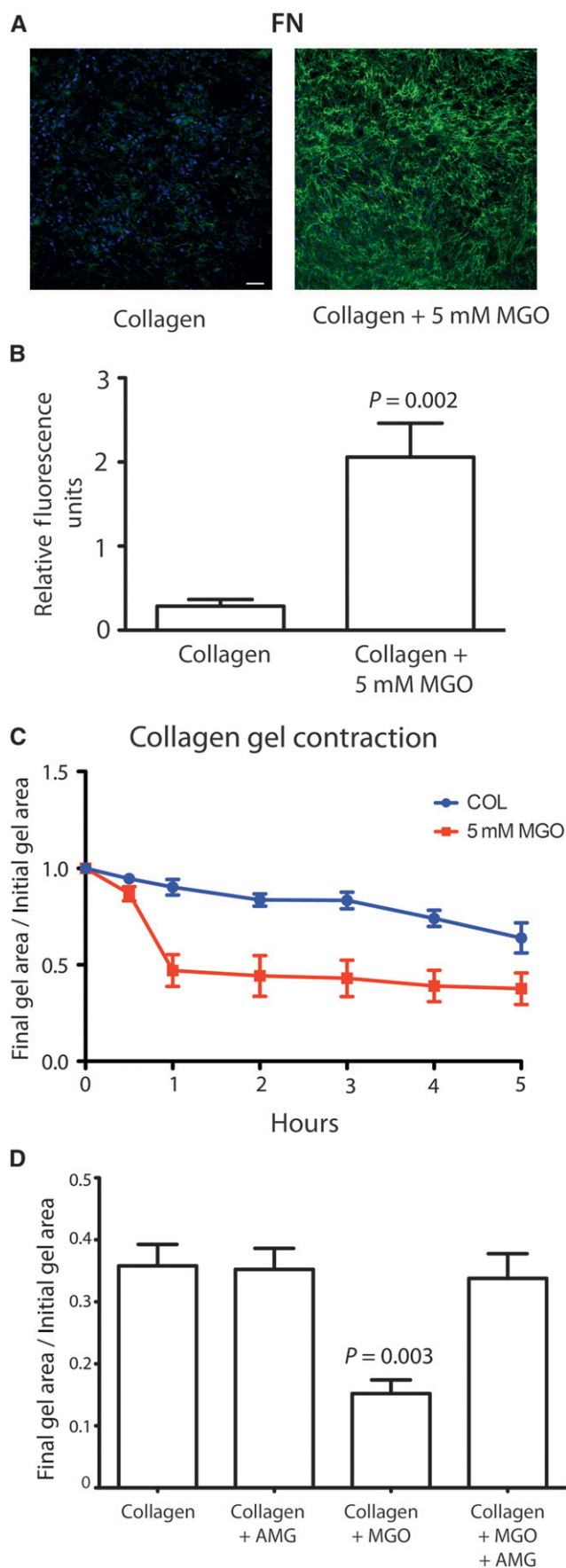
Figure 4.
*MGO-treated collagen stimulates FN protein levels and assembly. **A)** FN distribution assessed through immunofluorescence (green). Nuclei were stained with DAPI. Bar = 40 μm. Original magnification ×10. **B)** FN protein levels assessed through Western blot of DOC-soluble or DOC-insoluble cell lysates. **C)** Graphs represent average and SE of FN protein levels normalized against vinculin for DOC-soluble and -insoluble lysates and total FN. P values indicate differences between collagen and MGO-treated collagen conditions. **D)** FN protein levels measured through ELISA in cell-conditioned media of fibroblasts cultured over MGO or MGO-treated collagen. **E)** HGFs seeded over collagen or 5 mM MGO-treated collagen in presence or absence of the triple-helical peptide GFOGER or its control GPP-10 and DOC-soluble or -insoluble lysates analyzed through Western blot for FN. Vinculin was used as a loading control. **F)** Graph represents average and SE for FN protein levels normalized against vinculin. P value indicates significant differences between MGO-treated collagen and MGO-treated collagen plus the GFOGER peptide. Col = collagen.*

α-SMA, secretion and assembly of FN, and contraction of the surrounding collagen network. In addition, assembly of FN was dependent on recognition of the collagen-binding domain for integrins GFOGER, suggesting involvement of these collagen receptors in the organization of this response. These findings strongly suggest that MGO-glycated collagen stimulates differentiation of myofibroblasts in gingival connective tissue cells that may contribute to the abnormal wound-healing response in tissues exposed to MGO. This may be particularly important in the case of tissues modified by DM or aging where defect in connective tissue repair has been described.[27,28]

Myofibroblasts play a key role during wound healing where they secrete and organize the regenerating ECM during connective tissue healing.[15] However, abnormal differentiation or function of myofibroblasts has been associated with delayed or pathologic wound repair.[15,17] A number of studies have analyzed the effect of glycated collagen on connective tissue cells of the myocardium and periodontium.[3,11,18,29] Culture of human cardiac fibroblasts over glycated collagen stimulates differentiation of myofibroblasts through involvement of TGF-β2 and collagen-binding integrin α11β1.[18,29] In the present study, significant increase in the myofibroblastic marker α-SMA was observed in cells cultured over MGO-treated collagen compared with non-treated collagen. Although soluble TGF-β1 clearly exerted a more powerful effect on myofibroblastic differentiation compared with MGO-treated collagen, the nature and characteristics of these two sources of stimuli are quite different. Active TGF-β1 is released from ECM through a complex activation process that involves changes in local pH, proteolytic enzymes, and integrins.[15,17] Moreover, no in vivo measurements have been performed to identify the time frame during which TGF-β1 may be active in tissues. On the other hand, it is possible that MGO-modified collagen might remain in tissues for extended periods of time. In fact, it has been shown that remodeling of MGO-treated collagen is delayed in HGFs.[10] Therefore, it is possible to propose that glycated collagen might exert a chronic effect in tissues modifying the differentiation of cells for undetermined periods of time. It is important to highlight that gingival fibroblasts represent a heterogeneous population of cells and differentiation of myofibroblasts may vary according to the origin of

Figure 5.
*MGO stimulates collagen gel remodeling. **A)** FN distribution (green) visualized through confocal microscopy. Nuclei were stained with DAPI. Bar = 30 μm. Original magnification ×4. **B)** Graph represents quantification of FN immunofluorescence signal detected in three independent experiments. P value indicates significant differences between collagen and MGO-treated collagen conditions. **C)** Graph represents average and SE of collagen gel area. **D)** Graph represents average and SE of three independent experiments. P value indicates significant differences between collagen and MGO-treated collagen gels.*

the sample and cell culture conditions.[30,31] Clearly, in vivo studies are needed for precise evaluation of the differentiation of myofibroblasts in conditions characterized by glycation of ECM proteins such as collagens.

Regulation of FN in DM is critically important because this protein plays crucial roles in cell adhesion, cell migration, proliferation, and fibrosis.[32] In the skin of diabetic patients, increased levels of FN have been detected at the capillaries and dermo-epidermal basement membranes[33] and in the heart and kidney of rats with streptozotocin-induced DM.[34] Other studies have identified that soluble MGO stimulates FN expression in renal mesangial cells.[35] Moreover, injection of soluble MGO into the peritoneal cavity in rats induces an inflammatory response followed by fibrosis.[4] In this regard, observations revealed a potent effect of MGO-treated collagen on FN protein levels and assembly. Biologic effects may derive from this change in content and organization of this glycoprotein.[36] Increased assembly of FN molecules stimulates deposition of Type I collagen fibrils, promoting development of fibrosis.[21] Another potential effect derived from increased FN assembly relates to the turnover of this glycoprotein. FN remodeling is essential for proper wound healing.[37] This is critically important for cell migration and tissue remodeling where FN-binding integrins and matrix metalloproteinases are actively involved.[38] Therefore, it is possible that a more complex and densely packed FN network might alter the normal evolution of cell migration. It is interesting to consider that MGO may also glycate FN, altering the organization of this glycoprotein in tissues exposed to this glucose-derived product.[11]

Changes in the mechanical properties of collagen are relevant because increased levels of rigidity of ECM may stimulate FN assembly and myofibroblastic differentiation.[15,39,40] Importantly, skin of diabetic patients is characterized by altered mechanical properties.[41] To identify changes in the mechanical properties of collagens, the Young modulus was analyzed at the level of collagen gels and at individual collagen fibrils through AFM. Observations revealed

a decrease by a factor of two in the modulus of MGO-treated collagen fibrils. However, these changes were not noticeable at the macroscopic scale of collagen gels. Therefore, a mechanical influence on differentiation of myofibroblasts or production and assembly of FN was not demonstrated by the present study. Glycation of collagen by glucose-derived products are probably responsible for deterioration of tissue functions during aging and along evolution of DM.[42] In this regard, it is interesting to consider that many AGEs found in dermal collagen samples that include glucosepane, MGO, glyoxal, and carboxyethyl-lysine were recently associated with long-term microvascular complications in patients with type 1 DM.[13] From this group of glucose-derived products, glucosepane has been implicated as an important source of collagen crosslinking.[42,43] Future studies are needed to clarify the role of these compounds in the biologic response of collagen-binding cells such as fibroblasts.

During this study, MGO-treated collagen stimulated contraction of collagen gels. A number of cell functions may influence outcome of collagen gel contraction assays, including cell migration, collagen degradation, cell contraction, and structural modifications of the collagen molecule.[44] In experiments in the current study, many factors may have influenced contraction of collagen gels. Induction of α-SMA has been associated with increased cell contraction.[15,17] FN can also stimulate collagen gel remodeling.[45] Collagen glycation might also stimulate cross-linking of this molecule.[42,43] Therefore, the possibility that many of these factors might have stimulated collagen contraction cannot be ruled out. More studies are needed to clarify this point.

CONCLUSIONS

Collectively, the present study findings may help to explain that modification in the collagen matrix induced by MGO stimulates differentiation of myofibroblasts and upregulation and assembly of FN that alters the normal organization of connective tissue phenotypes and ECM components. This may be particularly relevant in the case of DM and aging where abnormal tissue organization and function may finally alter tissue homeostasis and response to injury.

ACKNOWLEDGMENTS

REFERENCES

1. Cooper RA, Anderson A. The formation and catabolism of methylglyoxal during glycolysis in *Escherichia coli*. *FEBS Lett* 1970;11:273-276.
2. Ahmed N, Thornalley PJ, Dawczynski J, et al. Methylglyoxal-derived hydroimidazolone advanced glycation end-products of human lens proteins. *Invest Ophthalmol Vis Sci* 2003;44:5287-5292.
3. Yuen A, Laschinger C, Talior I, et al. Methylglyoxal-modified collagen promotes myofibroblast differentiation. *Matrix Biol* 2010;29:537-548.
4. Onishi A, Akimoto T, Urabe M, et al. Attenuation of methylglyoxal-induced peritoneal fibrosis: Immunomodulation by interleukin-10. *Lab Invest* 2015;95:1353-1362.
5. Fleming TH, Theilen TM, Masania J, et al. Aging-dependent reduction in glyoxalase 1 delays wound healing. *Gerontology* 2013;59:427-437.
6. Kashket S, Maiden MF, Haffajee AD, Kashket ER. Accumulation of methylglyoxal in the gingival crevicular fluid of chronic periodontitis patients. *J Clin Periodontol* 2003;30:364-367.
7. Popova SN, Lundgren-Akerlund E, Wiig H, Gullberg D. Physiology and pathology of collagen receptors. *Acta Physiol (Oxf)* 2007;190:179-187.
8. Lo TW, Westwood ME, McLellan AC, Selwood T, Thornalley PJ. Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin. *J Biol Chem* 1994;269:32299-32305.
9. Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem* 2000;275:35-40.
10. Chong SA, Lee W, Arora PD, et al. Methylglyoxal inhibits the binding step of collagen phagocytosis. *J Biol Chem* 2007;282:8510-8520.
11. Murillo J, Wang Y, Xu X, et al. Advanced glycation of type I collagen and fibronectin modifies periodontal cell behavior. *J Periodontol* 2008;79:2190-2199.
12. Retamal IN, Hernández R, González-Rivas C, et al. Methylglyoxal and methylglyoxal-modified collagen as inducers of cellular injury in gingival connective tissue cells. *J Periodontal Res* 2016;51:812-821.
13. Genuth S, Sun W, Cleary P, et al; DCCT/EDIC Research Group. Skin advanced glycation end products glucosepane and methylglyoxal hydroimidazolone are independently associated with long-term microvascular complication progression of type 1 diabetes. *Diabetes* 2015;64:266-278.
14. Skalli O, Schürch W, Seemayer T, et al. Myofibroblasts from diverse pathologic settings are heterogeneous in their content of actin isoforms and intermediate filament proteins. *Lab Invest* 1989;60:275-285.
15. Klingberg F, Hinz B, White ES. The myofibroblast matrix: Implications for tissue repair and fibrosis. *J Pathol* 2013;229:298-309.
16. Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-β 1 induces α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993;122:103-111.

17. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: One function, multiple origins. *Am J Pathol* 2007;170:1807-1816.

18. Talior-Volodarsky I, Connelly KA, Arora PD, Gullberg D, McCulloch CA. α11 Integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. *Cardiovasc Res* 2012;96:265-275.

19. Singer II, Kawka DW, Kazazis DM, Clark RA. In vivo co-distribution of fibronectin and actin fibers in granulation tissue: Immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface. *J Cell Biol* 1984;98:2091-2106.

20. Muro AF, Moretti FA, Moore BB, et al. An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *Am J Respir Crit Care Med* 2008;177:638-645.

21. Sottile J, Shi F, Rublyevska I, Chiang HY, Lust J, Chandler J. Fibronectin-dependent collagen I deposition modulates the cell response to fibronectin. *Am J Physiol Cell Physiol* 2007;293:C1934-C1946.

22. Chevallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. *Nat Protoc* 2006;1: 1852-1858.

23. Sechler JL, Takada Y, Schwarzbauer JE. Altered rate of fibronectin matrix assembly by deletion of the first type III repeats. *J Cell Biol* 1996;134:573-583.

24. Sirghi L, Rossi F. Adhesion and elasticity in nanoscale indentation. *Appl Phys Lett* 2006;89:243118-243120.

25. Johnson KL, Kendall K, Roberts AD. Surface energy and the contact of elastic solids. *Proc R Soc Lond A* 1971;324:301-313.

26. Dobler D, Ahmed N, Song L, Eboigbodin KE, Thornalley PJ. Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification. *Diabetes* 2006;55:1961-1969.

27. Desta T, Li J, Chino T, Graves DT. Altered fibroblast proliferation and apoptosis in diabetic gingival wounds. *J Dent Res* 2010;89:609-614.

28. Cáceres M, Oyarzún A, Smith PC. Defective wound healing in the aging gingival tissue. *J Dent Res* 2014; 93:691-697.

29. Talior-Volodarsky I, Arora PD, Wang Y, et al. Glycated collagen induces α11 integrin expression through TGF-β2 and Smad3. *J Cell Physiol* 2015;230:327-336.

30. Giannopoulou C, Cimasoni G. Functional characteristics of gingival and periodontal ligament fibroblasts. *J Dent Res* 1996;75:895-902.

31. Smith PC, Martínez J. Differential uPA expression by TGF-β1 in gingival fibroblasts. *J Dent Res* 2006;85:150-155.

32. Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci* 2002;115:3861-3863.

33. Phan-Thanh L, Robert L, Derouette JC, Labat-Robert J. Increased biosynthesis and processing of fibronectin in fibroblasts from diabetic mice. *Proc Natl Acad Sci USA* 1987;84:1911-1915.

34. Roy S, Sala R, Cagliero E, Lorenzi M. Overexpression of fibronectin induced by diabetes or high glucose: Phenomenon with a memory. *Proc Natl Acad Sci USA* 1990;87:404-408.

35. Ho C, Lee PH, Huang WJ, Hsu YC, Lin CL, Wang JY. Methylglyoxal-induced fibronectin gene expression through Ras-mediated NADPH oxidase activation in renal mesangial cells. *Nephrology (Carlton)* 2007;12: 348-356.

36. Coelho NM, McCulloch CA. Contribution of collagen adhesion receptors to tissue fibrosis. *Cell Tissue Res* 2016;365:521-538.

37. Wolanska KI, Morgan MR. Fibronectin remodelling: Cell-mediated regulation of the microenvironment. *Biochem Soc Trans* 2015;43:122-128.

38. Hynes RO. The extracellular matrix: Not just pretty fibrils. *Science* 2009;326:1216-1219.

39. Halliday NL, Tomasek JJ. Mechanical properties of the extracellular matrix influence fibronectin fibril assembly in vitro. *Exp Cell Res* 1995;217:109-117.

40. Carraher CL, Schwarzbauer JE. Regulation of matrix assembly through rigidity-dependent fibronectin conformational changes. *J Biol Chem* 2013;288:14805-14814.

41. Bermudez DM, Herdrich BJ, Xu J, et al. Impaired biomechanical properties of diabetic skin implications in pathogenesis of diabetic wound complications. *Am J Pathol* 2011;178:2215-2223.

42. Avery NC, Bailey AJ. The effects of the Maillard reaction on the physical properties and cell interactions of collagen. *Pathol Biol (Paris)* 2006;54:387-395.

43. Biemel KM, Reihl O, Conrad J, Lederer MO. Formation pathways for lysine-arginine cross-links derived from hexoses and pentoses by Maillard processes: Unraveling the structure of a pentosidine precursor. *J Biol Chem* 2001;276:23405-23412.

44. Grinnell F, Petroll WM. Cell motility and mechanics in three-dimensional collagen matrices. *Annu Rev Cell Dev Biol* 2010;26:335-361.

45. Liu Y, Yanai R, Lu Y, Kimura K, Nishida T. Promotion by fibronectin of collagen gel contraction mediated by human corneal fibroblasts. *Exp Eye Res* 2006;83:1196-1204.

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