



Methylglyoxal-modified collagen promotes myofibroblast differentiation

Amy Yuen^a, Carol Laschinger^a, Ilana Talior^a, Wilson Lee^a, Matthew Chan^a, Juliana Birek^a,
Edmond W.K. Young^b, Konesh Sivagurunathan^b, Emily Won^b, Craig A. Simmons^b, C.A. McCulloch^{a,*}

^a CIHR Group in Matrix Dynamics, University of Toronto, Canada

^b Institute of Biomaterials and Biomedical Engineering, University of Toronto, Canada

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ABSTRACT

Fibrosis is a frequent complication of diabetes mellitus in many organs and tissues but the mechanism of how diabetes-induced glycation of extracellular matrix proteins impacts the formation of fibrotic lesions is not defined. As fibrosis is mediated by myofibroblasts, we investigated the effect of collagen glycation on the conversion of human cardiac fibroblasts to myofibroblasts. Collagen glycation was modeled by the glucose metabolite, methylglyoxal (MGO). Cells cultured on MGO-treated collagen exhibited increased activity of the α -smooth muscle actin promoter and enhanced expression of α -smooth muscle actin, ED-A fibronectin and cadherin, which are markers for myofibroblasts. In cells remodeling floating or stress-relaxed collagen gels, MGO treatment promoted more contraction ($p < 0.025$) than vehicle controls, which was MGO dose-dependent. Transwell assays showed that cell migration was increased by MGO-treated collagen ($p < 0.025$). In shear-force detachment assays, cells on MGO-treated collagen were less adherent than untreated collagen, and the formation of high affinity, $\beta 1$ integrin-dependent adhesions was inhibited. MGO-collagen-induced expression of SMA was dependent on TGF- β but not on Rho kinase. We conclude that collagen glycation augments the formation and migration of myofibroblasts, critical processes in the development of fibrosis in diabetes.

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1. Introduction

Heart failure is a high prevalence disorder (Fedak et al., 2005) of substantial morbidity (Heineke and Molkentin, 2006) that is common in diabetics (Kannel et al., 1974). Cardiovascular complications are strongly associated with diabetes-related mortality (Jay et al., 2006) but the contribution of hyperglycemia-induced changes of the cardiac extracellular matrix to diabetic cardiomyopathy has not been defined. Hyperglycemic episodes cause increased glycation of extracellular matrix proteins, including collagens. Non-enzymatic processes such as the Maillard reaction (Aronson, 2003) mediate the formation of advanced glycation end products and increased collagen cross-linking (Avery and Bailey, 2006). These processes can alter cell adhesions to collagen (Chong et al., 2007) and its biomechanical functions (Paul and Bailey, 1996).

The cardiac interstitium is populated by fibroblasts, which comprise two-thirds of the cell population of the ventricular wall (Camelliti et al., 2006). Homeostasis of the cardiac interstitium requires precise regulation of matrix remodeling and tight control of

the migration, proliferation and differentiation of cardiac fibroblasts (Fedak et al., 2005). The differentiation of fibroblasts into myofibroblasts is strongly upregulated in failing hearts (Brown et al., 2005; Weber and Sun, 2000; Baudino et al., 2006) and is characterized by *de novo* expression of α -smooth muscle actin (Gabbiani et al., 1971) and by increased formation of a disorganized collagen matrix (Sappino et al., 1990).

Currently, very little is known about how post-translational modifications to collagen influence myofibroblast differentiation. We treated collagen with methylglyoxal, which is widely used (Chong et al., 2007; Paul and Bailey, 1999; Dobler et al., 2006; Goh and Cooper, 2008; Pedchenko et al., 2005; Shamsi et al., 1998) to model the effect of diabetes-induced glycation of the extracellular matrix on cell function. Recent data show that methylglyoxal, which is a major component of the diabetic environment and is involved in carbonyl stress, has an important impact on cell adhesion that affects RGD and GFOGER residues in collagen that bind to integrins (Dobler et al., 2006; Pedchenko et al., 2005; Pozzi et al., 2009). These modifications have critical effects on cell adhesion-related functions. In the context of cardiac fibroblast differentiation into myofibroblasts, our main findings are that collagen glycation inhibits the formation of high affinity cell adhesions, while enhancing the expression of α -smooth muscle actin, ED-A fibronectin and cadherin, and cell migration over collagen. These data suggest a novel mechanism for cardiac fibrosis in which

* Corresponding author. Room 244, Fitzgerald Building, University of Toronto, 150 College Street, Toronto, ON, Canada, M5S 3E2. Tel.: +1 416 978 1258; fax: +1 416 978 5956.

E-mail address: christopher.mcculloch@utoronto.ca (C.A. McCulloch).

glycated collagen inhibits cell–matrix attachments while indirectly promoting the development of intercellular adhesions and enabling myofibroblast differentiation.

2. Material and methods

2.1. Reagents

Mouse monoclonal antibodies to type I bovine collagen (clone 1319), α -SMA (clone 1A4), desmin (clone DE-U-10), vinculin and vimentin (clone VIM 13.2) were purchased from Sigma-Aldrich (Oakville, ON). Antibody to human pro-collagen type I was obtained from J. Sodek, University of Toronto. Antibody to methylglyoxal-AGE (Arg-pyrimidine) was from Biologo (Germany). Mouse monoclonal antibody to paxillin (clone 5H11) was obtained from Upstate (Lake Placid, NY). Mouse monoclonal blocking antibody to β 1 integrin (clone 4B4) was purchased from Beckman-Coulter (Burlington, ON). Antibodies to activated β 1 integrin (clone 12G10) and to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Chemicon-Millipore (Billerica, MA). Antibody to pan-cadherin (CH-19) was from Abcam (Cambridge, MA) and to β -catenin was from Transduction-BD Biosciences (Mississauga, ON) and to ED-A fibronectin from Serotec (Oxford, UK). Neutralizing antibody to TGF- β 1 was from R&D (Minneapolis, MN). Transwell 8.0 μ m pore size polycarbonate membranes were obtained from Corning Inc. (Corning, NY). Purified, pepsin-digested, type I calf skin collagen was from Inamed (Fremont, CA).

2.2. Collagen glycation

Methylglyoxal (MGO) was used to glycate collagen (Dobler et al., 2006; Goh and Cooper, 2008; Pedchenko et al., 2005; Shamsi et al., 1998) instead of glucose or ribose as this procedure reduces the time required for collagen glycation and facilitates more consistent cross-linking of collagen gels (Paul and Bailey, 1999). Collagen gels were incubated overnight with 1 mM, 0.1 mM or 0.01 mM MGO at 37 °C and rinsed with PBS as described (Chong et al., 2007).

2.3. Mechanical characteristics of collagen gels

The effect of MGO-induced glycation on the rigidity of collagen gels was examined using *in situ* scanning probe microscopy. Force spectroscopy data were acquired on a Digital Instruments Nanoscope IIIa Bioscope scanning probe microscope using the Nanoscope software to control the Z-directed piezo motion. The force curves were acquired using the Bioscope fluid cell using 115 μ m long oxide-sharpened silicon nitride V-shaped cantilevers (model DNP-S, Veeco, Plainview, NY; 35° half angle) with a measured force constant (k), as determined from the thermal resonance peak of the cantilever of ~ 0.06 N/m ($k \sim (k_B T) / \langle A^2 \rangle$) on samples placed in plastic well plates and immersed in buffer solution. The vertical deflection of the AFM tip was monitored separately at a sampling frequency of 60 kHz using a National Instruments DAQ card (model PCI-6251) and in-house written LabView software (version 7.1, National Instruments). All force curves were acquired at a tip approach rate of ~ 4 μ m/s and were collected continuously. During the force curve measurement, the maximum force applied to the sample surface by the tip was ~ 0.625 nN, as defined by the force curve deflection trigger set-point. Ten force curves were collected at one or two locations on each gel and averaged. AFM force–displacement curves were fit to a Hertz contact model (Dimitriadis et al., 2002) by nonlinear regression (NLREG Version 6.3 Advanced). Elastic moduli of the gels were estimated based on the curve fits for the portion of the curve up to 200 nm of indentation, assuming a Poisson's ratio of 0.5.

2.4. Cell culture

Human cardiac fibroblasts (HCF, ScienCell, Carlsbad, CA) were plated in DMEM medium containing fetal bovine serum, fibroblast growth supplement (ScienCell) and penicillin/streptomycin (100 U/ml and 100 μ g/ml). Cells were maintained at 37 °C in a humidified incubator containing 5% CO₂ and passaged with trypsin and EDTA. For experiments on the effect of MGO-collagen on cell behavior, cells were plated at 3.0×10^4 cells per 28 mm² of collagen substrate (low density) or, for most experiments, at 6.0×10^4 cells per 28 mm² of collagen substrate (high density). DDR1 null fibroblasts (generously provided by W. Vogel, University of Toronto) were cultured in α -minimal essential medium containing 10% (v/v) fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μ g/ml). β 1 integrin null mouse fibroblasts (GD25 cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin–streptomycin and puromycin (10 μ g/ml).

2.5. Fibroblast phenotype

Human cardiac fibroblasts were immunostained for vimentin, SMA, type I collagen and desmin (Sappino et al., 1990). Passage-3 cultures were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with primary antibodies and counter-stained with FITC-conjugated goat anti-mouse antibody. Cell nuclei were stained with DAPI or propidium iodide and imaged by fluorescence or confocal microscopy (SP, Leica, Germany).

2.6. Collagen gels

The effect of glycation on collagen gel contraction was examined in floating and stress-relaxed collagen gels. Sterile collagen gels (1.875 mg/ml) were polymerized at 37 °C and treated with MGO or PBS overnight. Gels were rinsed before plating with cells on the upper gel surface. To optimize plating densities for collagen gel contraction experiments, assays were performed at cell densities ranging between 10^4 and 10^7 cells/ml. Contraction of floating collagen gels was measured daily for 4 days by microscopy using an intraocular grid to estimate gel diameters. MGO and control collagen gels were initially of the same diameter, prior to contraction assays. For stress-relaxed collagen gels, collagen matrices were polymerized for 60 min at 37 °C and cells were plated on top of the collagen gels at 10^7 cells/ml of collagen solution. To initiate contraction, mechanically stressed matrices were gently released from the culture dish with a spatula.

2.7. Analysis of cross-linked collagen

SDS-PAGE sample buffer was added to control or MGO-treated collagen. Samples were heated to 50 °C for 30 min and analyzed by SDS-PAGE (7.5% acrylamide) followed by silver staining.

2.8. SMA expression and stress fiber organization

As fibroblasts respond to endogenously generated forces by re-organization of actin filaments (Petroll et al., 1993) and increased expression of SMA (Gabbiani, 1999), we determined whether collagen glycation affects SMA expression and actin filament organization. Cells were plated on control or glycated collagen gels. After fixation, cells were immunostained for SMA or stained for actin filaments with rhodamine phalloidin.

2.9. Cell morphology

Scanning electron microscopy was used to visualize cell morphology. Gels were fixed in formaldehyde on poly-L-lysine-coated glass slides and dehydrated through an ethanol series. The samples were

critical point dried in a Polaron CPD7501. Samples were mounted on aluminum stubs and plasma sprayed with a 5 nm thick coat of platinum in a Polaron SC 515 SEM coating system and examined with a Hitachi C-2500 scanning electron microscope.

2.10. Migration assays

Migration assays were performed with Transwell chambers (8.0 μ m diameter pore size). Membranes were coated with collagen and incubated with either PBS or MGO (1 mM) overnight. To avoid clogging of the pores with collagen, vacuum was applied to the chambers. The patency of the pores was then verified by light microscopy after Coomassie blue staining. Cells (1×10^4) were seeded on top of the membrane insert in serum-free DMEM. In the bottom chamber, DMEM containing 10% FBS was used as a chemoattractant. After 24 h, cells were washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with DAPI. Migrating cells were counted on the lower surface of the membrane at 250 \times .

2.11. Adhesion assay

Adhesion strength under shear force was measured with parallel arrays of micro-channels mounted on glass slides (Young et al., 2007). The glass was coated with collagen and treated with PBS or MGO at different concentrations directly in the micro-channels. To measure relative adhesion strengths, cells were subjected to 110 dyn/cm² flow-induced shear stress for 4 min. The number of attached cells per unit area after shearing was quantified by ImageJ.

2.12. SMA expression

Cell lysates were prepared by adding buffer A (2% Triton X-100, 160 mM KCL, 40 mM Tris-HCl, 20 mM EGTA, 10 mM PMSF, 1 mM leupeptin, 1 mM benzamidine) and then an equal volume of Laemmli sample buffer. The protein content of samples was estimated with Bio-Rad assays. Equal amounts of protein were separated on 10% SDS gels, transferred to nitrocellulose filters blocked with milk, incubated with SMA antibody, horseradish peroxidase-conjugated second antibody, and developed with ECL reagents (Amersham, Oakville, ON). The equivalence of protein loading of individual samples was examined by immunoblotting for GAPDH.

2.13. Apoptosis

FITC-annexin binding (Roche, Montreal, QB) was used to identify apoptotic cells in cultures of fibroblasts growing on MGO- or untreated collagen.

2.14. Analysis of SMA promoter activity

Cells were transiently transfected with a luciferase reporter plasmid for SMA obtained from R. Nemenoff (Denver, CO). Cells were co-transfected with a β -galactosidase Rous sarcoma virus (RSV) expression plasmid to normalize for equal loading using LipofectAMINE 2000 (Invitrogen). Following transfection (20 h), cells were plated on PBS or MGO-treated collagen and grown for 24 h. Cell extracts were prepared with a detergent lysis method and luciferase assays and β -galactosidase reporter enzyme activity were measured.

2.15. Collagen bead-associated proteins

Collagen-coated magnetite beads were incubated with an acidic solution of collagen (3 mg/ml, 1 ml) and neutralized with NaOH (1 N, 100 μ l) to initiate fibril formation. Beads were vortexed, incubated at

37 °C for 10 min, pelleted, incubated with vehicle or MGO, sonicated for 10 s, and incubated overnight at 37 °C in a humidified incubator containing 5% CO₂. BSA coated beads were prepared by incubating latex beads with 1 ml of 1% (w/v) BSA for 30 min at 37 °C. The beads were pelleted, incubated with control or MGO-containing medium (see below), sonicated in solution for 10 s, and incubated overnight at 37 °C in a humidified incubator containing 5% CO₂. After incubation, beads were pelleted, washed with PBS and re-suspended in PBS prior to incubation with cells for 1 h. For immunoblotting of bead-associated proteins, cells were washed with PBS, lysed and the beads were electronically counted. Equal numbers of beads from each sample were boiled and bead-associated proteins were separated on 10% SDS gels, transferred to nitrocellulose filters and immunoblotted for SMA as described above.

2.16. Quantitative real-time PCR

Human cardiac fibroblasts were plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) overnight, or for 2 or 5 days. RNA was isolated using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). cDNA was generated using Reverse Transcriptase (Fermentas, Burlington, ON, Canada). Real-time PCR was performed using iQTMSYBR[®] Green Supermix (Bio-Rad, Hercules, CA) with validated human collagen-I primers: F'-TACCATGACCGAGACGTGTGGAAA; R'-AGATCAGTCATCG-CACAACACCT; or with α -smooth muscle actin primers: F'-TGA-CAATGGCTCTGGGCTCTGTAA; R'-TTCGTCACCCACGTAGCTGTCTTT; or with P-cadherin primers: F'-ATGACGTGGCACCAACCAT; R'-GTTAGCCGCTTCAGTTCTC; or with TGF- β 1 primers: F'-CCCAG-CATCTGCAAAGCTC; R'-GTCAATGTACAGCTGCCGCA; or with TGF- β 2 primers: F'-TAACATCTCCAACCCAGCGCTACA; R'-GCATCAGTTACATC-GAAGGAGAGCCA; or with TGF- β 3 primers: F'-ACTGGCTGTCTGCCC-TAAAGGAAT; R'-AAGACCCGGAATTCTGCTCGGAATA.

Relative quantification was calculated using the $\Delta\Delta$ Ct method normalized to human GAPDH using the primers (F'-AATCCCATCAC-CATCTTCCA; R'-TGGACTCCACGACTACTCA).

2.17. β 1 integrin activation

As the α 2 β 1 integrin is the principal collagen receptor that mediates collagen gel contraction (Schiro et al., 1991), we measured the effect of collagen glycation on β 1 integrin activation by immunostaining with 12G10, which recognizes a neo-epitope in activated β 1 integrins (Mould et al., 1995).

2.18. Statistical analysis

For all data sets, experiments were repeated at least three times and each repeat contained three replicates. For continuous variables, means and standard errors of the mean were computed. Comparisons between two groups were made with the unpaired Student's *t*-test. For multiple comparisons ANOVA with *post-hoc* Bonferroni analysis was performed. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Collagen glycation and cardiac fibroblast phenotype

We modeled diabetes-induced alteration of the extracellular matrix with the glucose metabolite methylglyoxal (MGO), which mediates collagen glycation (Chong et al., 2007). MGO induced the formation of both higher and lower molecular mass collagen molecules (Fig. 1A), reflecting the possible presence of MGO-collagen degradation products and consequent variations of electrophoretic mobility. Immunoblotting of collagen with an antibody that recognizes MGO-advanced glycation end products (Arg-pyrimidine) showed that MGO treatment of collagen strongly increased glycation

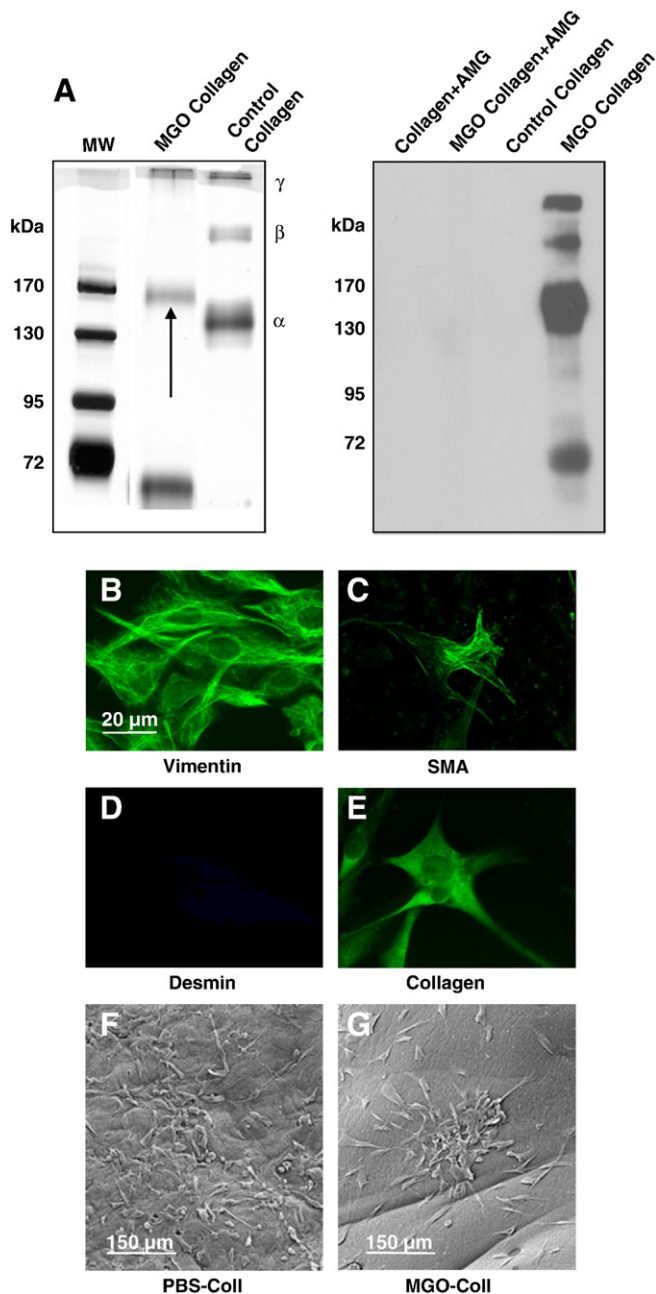


Fig. 1. (A) SDS-PAGE analysis of control and MGO-treated collagen. Note the higher and lower molecular mass aggregates that are detected as a result of glycation-induced adduct formation and alteration of charge profiles of collagen molecules due to MGO treatment. Immunoblot analysis of collagen, 1 mM MGO-treated collagen, 20 mM aminoguanidine and 1 mM MGO-treated collagen, or 20 mM aminoguanidine-treated collagen. All collagen samples were initially attached to cell culture plates. Collagen was solubilized by hot SDS-PAGE sample buffer. Samples were separated by electrophoresis, transferred to a nitrocellulose membrane and immunoblotted for Arg-pyrimidine residues. (B–E) Fluorescence micrographs of human cardiac fibroblasts (passage 3) cultured on glass coverslips overnight and immunostained for vimentin, SMA, desmin and pro-collagen. (F,G) Scanning electron micrographs of human cardiac fibroblasts plated on PBS-treated collagen gels and 1 mM MGO-treated collagen gels. Cells plated on MGO-treated collagen did not attach as well as controls and exhibited lower plating densities.

(Fig. 1A), which was blocked by aminoguanidine. As myofibroblast differentiation is affected in part by the stiffness of the substrate (Arora et al., 1999), we characterized the mechanical properties of collagen gels by atomic force microscopy. We found no differences in the elastic moduli of collagen gels treated with vehicle or MGO at different concentrations (data not shown).

Phenotyping of human cardiac fibroblasts by immunostaining showed that in passage 3 cells that had been maintained on tissue culture plastic for 2 days, 90% and 70% exhibited staining for vimentin and pro-collagen respectively, but there was no staining for desmin (Fig. 1B–E); ~20% of the cells stained for SMA. Scanning electron micrographs of human cardiac fibroblasts plated at identical densities on PBS-treated collagen gels or 1 mM MGO-treated collagen gels showed that cells plated on MGO-treated collagen were clustered and not evenly distributed over the gels (Fig. 1F,G).

3.2. Collagen gel contraction

We determined the optimal cell densities for the stress-relaxed and floating gel contraction assays (Grinnell, 2003) to analyze the effect of MGO treatment on collagen contraction. For stress-relaxed assays in which measurements were made over 2–3 h, a density of 10^7 cells/ml was optimal while in contrast, for floating collagen gels, we used densities of 10^6 cells/ml since at this concentration the changes of gel diameter were most readily measured over a 4 day sampling period. Cells were incubated on gels so that equivalent numbers of cells were attached for collagen and MGO-collagen conditions, although the distribution of the cells on the gels was not homogeneous on glycated collagen (Fig. 1). For the stress-relaxed contraction assays, measurements over 2.5 h showed that 1 mM MGO-treated collagen gels contracted significantly more than untreated controls (Fig. 2A: $p < 0.05$, slope of best fit linear curve for 1 mM MGO-treated collagen (slope = 4.0) was 1.2 fold higher than PBS-collagen (3.3)). There were similar differences attributable to MGO in the floating collagen gel assays (Fig. 2B: $p < 0.025$; slope of best fit linear curve for 1 mM MGO-treated collagen (4.5) was 1.4 times higher than PBS-collagen (3.1)).

3.3. SMA expression

As there was enhanced contraction of MGO-treated collagen gels compared to controls, we examined whether there was an increase of the percentage of cells expressing α -smooth muscle actin (SMA) since SMA expression by fibroblasts is associated with enhanced collagen gel contraction (Arora and McCulloch, 1994). In cells incubated on control collagen or MGO-collagen substrates for 2 days, there were equivalent numbers of SMA-stained cells in the different conditions but there were relatively fewer cells that did not express SMA on the MGO-treated collagen. Consequently, there were higher relative proportions of SMA-stained cells as a result of MGO treatment (Fig. 3A). In these cultures, SMA was incorporated into well-developed bundles of actin filaments that appeared to be connected to adjacent, possibly contacting cells (Fig. 3B). We immunoblotted cultures for SMA and GAPDH (as loading control) in cells plated on collagen or MGO-collagen and cultured for 2 days. Even at low concentrations of MGO used for treatment of collagen, there was more SMA in cells that had been plated on MGO-collagen than on PBS-treated collagen (Fig. 3C; quantification of immunoblot ratios of SMA to GAPDH). Some of the increased SMA expression was likely due to enhanced SMA transcription since cells plated on MGO-collagen for 2 days showed 1.6-fold higher ($p < 0.02$) SMA luciferase activity than cells plated on control collagen (data were normalized to a loading control plasmid, RSV) (Fig. 3D). Notably, quantification of immunoblots for SMA and GAPDH followed by densitometry indicated that plating cells on MGO-collagen that were co-incubated with 20 mM aminoguanidine (a treatment that prevents the formation of glycosylated proteins) (Lo et al., 1994) including collagen (Chong et al., 2007), inhibited the MGO-collagen-induced enhancement of SMA expression (Fig. 3E). The effect of aminoguanidine on MGO-collagen treatment indicated that the enhanced expression of SMA by MGO was likely due to collagen glycation.

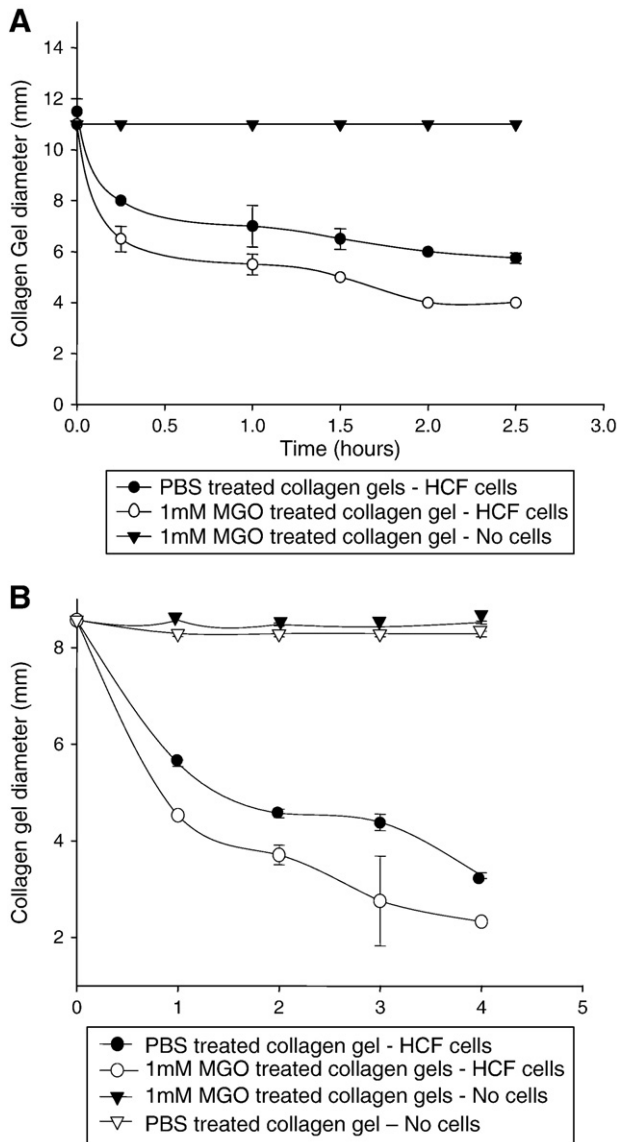


Fig. 2. (A) Stress-relaxed collagen gel contraction assays for control and MGO-collagen. Each data point is the mean \pm standard errors of the mean and the data are based on 4 replicates. (B) Mean \pm standard errors from floating collagen gels for control and MGO-collagen.

As a result of the relatively smaller percentages of non-SMA-expressing cells that adhered to MGO-collagen, we considered that the increased percentage of cells expressing SMA may be attributable to differential susceptibility to apoptosis in the SMA-negative and SMA-expressing cell populations. Accordingly, we quantified apoptosis by FITC-annexin binding, a marker of early stages of apoptosis in 1 day cultures. One day cultures were used instead of 2 day cultures because dying cells detached from the substrate could not be accurately analyzed for apoptosis and SMA staining. For the whole cell population there were increased percentages of annexin-positive cells when plated on MGO-collagen (Fig. 3F; all MGO-collagen conditions were $p < 0.01$ compared to PBS controls). In cells plated for 1 day on 0.1 M MGO-collagen, stained with FITC-annexin and immunostained for SMA, there was a significantly lower percentage of annexin-positive cells expressing SMA compared to SMA-null cells ($3.1 \pm 2.2\%$ versus $28.2 \pm 14.1\%$; $p < 0.05$). In contrast, for cells plated on control collagen there were equivalent % of annexin-positive SMA-expressing cells and SMA-negative cells (annexin-positive cells: SMA-expressing cells = 8.23 ± 4.27 ; SMA-null cells = $5.4 \pm 5.4\%$; $p > 0.2$). These data indicate that culture of human

cardiac fibroblasts on MGO-treated collagen favors survival of SMA-expressing cells.

Quantitative real-time PCR showed that culture on glycosylated collagen increased mRNA expression of α -SMA (Fig. 4A) and that over time in culture there was an increase of the expression of collagen-1, a myofibroblast marker (Fig. 4B). We found no detectable N-cadherin mRNA by real-time PCR (data not shown) but there was increased expression of the mRNA for P-cadherin, which was detected after 5 days culture (Fig. 4C). Analysis of TGF- β isoform expression showed no detectable expression for TGF- β 1 (data not shown), a 2.5-fold increase of TGF- β 2 and a marked decrease of TGF- β 3 after 2 days of cell plating on MGO-collagen compared to collagen.

3.4. Migration

The observed differences of collagen gel contraction attributable to MGO described above (Fig. 2) could be due in part to differences in the rates of cell migration. In Transwell assays we found enhanced migration of cells plated on 1 mM MGO-treated collagen compared to vehicle controls and of cells plated on lower concentrations of MGO-collagen compared to control collagen (Fig. 5A; $p < 0.025$).

Migration of cells involves a complex series of cellular events and one of the rate-determining factors is adhesion strength to the substrate (Loftis et al., 2003). We compared adhesion strength of cells plated on control or MGO-collagen using a microfluidics flow chamber that generates prescribed shear force (Fig. 5B). Cells plated on control collagen were more strongly adherent than cells plated on all the MGO-treated collagen conditions ($p < 0.05$), suggesting that MGO treatment of collagen inhibits high affinity cell adhesions to collagen.

3.5. Collagen receptors

Since attachment of fibroblasts to collagen is mediated by β 1 integrins, we first quantified the effect of MGO treatment of collagen on the formation of high affinity β 1 integrins. Cells were immunostained with 12G10, an antibody that recognizes a neo-epitope in ligand-bound, activated β 1 integrins. Cells that were plated and allowed to spread on MGO-collagen showed no 12G10 staining at their leading edges compared to cells plated on vehicle-treated collagen (Fig. 6). Notably, staining for cortical actin (i.e. not associated with focal adhesions) was unchanged by plating cells on MGO-collagen.

The α 2 β 1 integrin is important for collagen gel contraction by fibroblasts (Schirow et al., 1991). As MGO treatment of collagen appeared to affect the contractile capacity of human cardiac fibroblasts, we determined the functional importance of the β 1 integrin in mediating the effects of MGO-collagen on gel contraction. Cells were pre-incubated with irrelevant antibody or a β 1 integrin-inhibiting antibody (4B4), which effectively blocks all β 1 integrin-forming heterodimers. Cells were then plated on MGO-treated or control floating collagen gels as described above. For antibody controls, there was faster contraction for cells cultured on MGO-treated collagen gels compared to control collagen ($p < 0.05$; Table 1). Treatment of cells with an antibody (4B4) that binds to and inhibits β 1 integrin interactions with collagen greatly reduced contractility of cells plated on control or MGO-collagen and the differences between the three conditions were not statistically significant ($p > 0.2$). We also examined whether the differences of contraction rates of cells plated on MGO-treated collagen or control collagen could be dissipated by an activating antibody to the β 1 integrin subunit. Cells were pre-incubated with a β 1 integrin-activating antibody (12G10) and plated on control collagen or MGO-treated collagen gels. There was faster contraction for cells cultured on 1 mM MGO-treated collagen gels than PBS-collagen controls ($p < 0.05$; Table 1) independent of the activating antibody treatment. These results were not due to reduced surface expression of β 1 integrins since flow cytometry analysis of non-permeabilized cells stained with the 4B4 antibody to detect cell

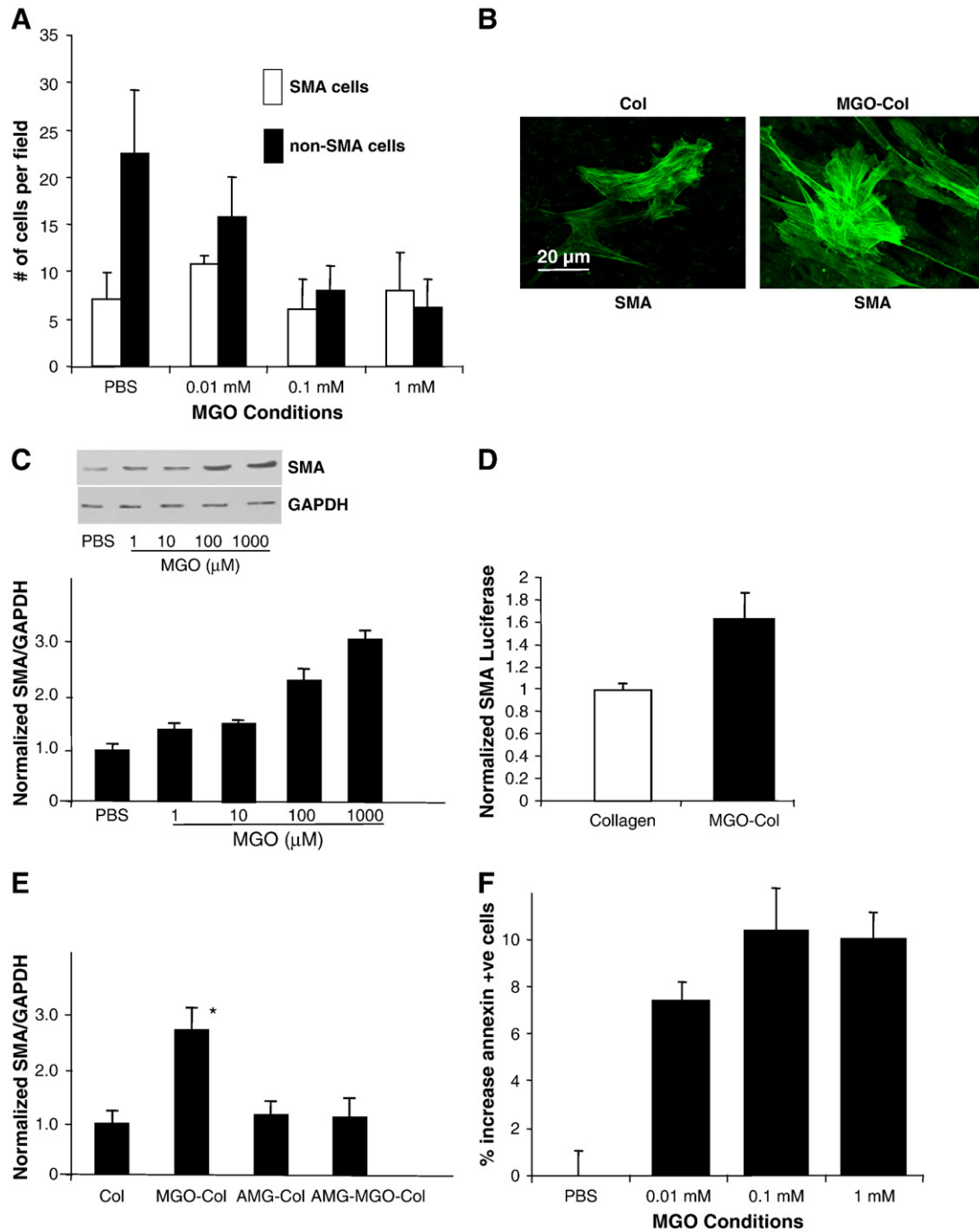


Fig. 3. (A) Morphometric analysis of cells immunostained for SMA. Cells were categorized as stained or not stained based upon fluorescence threshold intensities determined after staining with irrelevant antibody. Cells were plated on collagen or MGO-collagen gels for 2 days. Data are mean \pm standard error of number of cells counted in 250 \times power microscope fields (3 fields per culture from 4 separate cultures). (B) Cells immunostained for SMA after 2 days plating on collagen or 1 mM MGO-collagen. (C) Immunoblots of SMA and GAPDH of cells cultured on control collagen or MGO-collagen for 2 days at indicated concentrations of MGO treatment of collagen. Histogram shows mean \pm standard error of SMA:GAPDH blot densities for cells plated on PBS-treated collagen or MGO-collagen at the indicated MGO concentrations ($n = 3$ replicates per condition). Even at low concentrations of MGO (i.e. 1 μ M MGO) there were significant ($p < 0.05$) increases of SMA. (D) Cells were co-transfected with luciferase SMA promoter and β -galactosidase Rous sarcoma virus (RSV) constructs. RSV was used as a loading control. Data are mean \pm standard error of mean of normalized luciferase activity. Cells plated on MGO-collagen showed 1.6 fold increase of SMA promoter activity ($p < 0.05$). (E) Cells whether plated on collagen only, MGO-treated collagen, collagen co-incubated with aminoguanidine or MGO-treated collagen co-incubated with aminoguanidine. Quantification of SMA and GAPDH expression was measured by immunoblotting. The effect of aminoguanidine on MGO-collagen treatment indicated that enhanced expression of SMA by MGO was likely due to collagen glycation. * indicates $p < 0.01$. (F) Percentages of annexin-positive cells by fluorescence microscopy. Data are mean % increases \pm standard error of mean above % of annexin-positive cells plated on PBS-collagen.

surface integrin abundance showed no difference (cells on collagen – 14.0 ± 1.0 fluorescence units; cells on 0.1 mM MGO-collagen 12.7 ± 1.0 fluorescence units; $p > 0.2$). Collectively these findings indicate that the effect of MGO on collagen gel contraction is due in part to $\beta 1$ integrin interactions with collagen but does not require $\beta 1$ integrin activation.

We examined the effect of $\beta 1$ integrin function on SMA content in cells plated on control or MGO-treated collagen. Human cardiac fibroblasts exhibited increased SMA content when plated on MGO-collagen compared to control collagen (Fig. 7A). Similarly, after incubation with $\beta 1$ integrin-activating antibodies, there was increased SMA expression with MGO treatment of collagen (Fig. 7B) but there

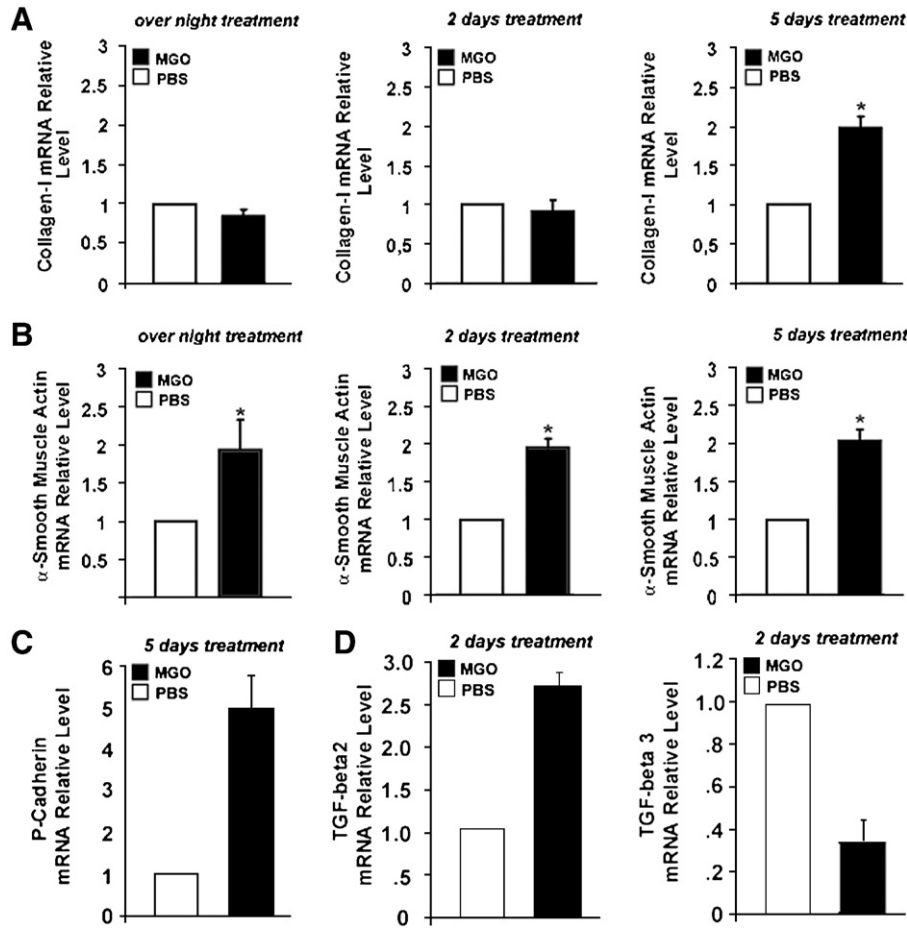


Fig. 4. MGO treatment increases mRNA expression for collagen-I, α -smooth muscle actin and P-cadherin. Human cardiac fibroblasts were plated on control collagen (PBS) or 1 mM MGO-collagen (MGO) for overnight, 2 days or 5 days. The effect of MGO on α -smooth muscle actin (A), collagen-I (B), P-cadherin (C) and TGF- β 2 and TGF- β 3 (D) mRNA levels were estimated by real-time quantitative PCR. For real-time PCR analysis, Ct values were GAPDH corrected and subjected to a *t*-test to determine statistical differences between samples. Data are the means \pm SE relative to PBS-collagen treated cells from 3 to 5 independent experiments; *, indicates $p \leq 0.05$.

was no detectable difference in SMA content between the untreated versus β 1 integrin-activating antibody, which corresponded to the collagen gel contraction data described above. When β 1 integrin-inhibiting antibodies were used, SMA expression was not detected, whether or not collagen was treated with MGO (Fig. 7C). We also examined SMA expression in cells which do not express β 1 integrins (GD25 cells (Fassler et al., 1995)). There was minimal SMA expression (Fig. 7D), confirming the role of the β 1 integrin in MGO-collagen-induced expression of SMA. As an additional control, we examined cells that did not express DDR1, an important but separate receptor for fibrillar collagens that acts independently of β 1 integrins (Vogel, 1999). These cells exhibited similar patterns of α -SMA expression after MGO treatment of collagen as controls (Fig. 7E), indicating that the β 1 integrin and not DDR1 was important in mediating the MGO-collagen effect on SMA expression.

3.6. Focal adhesions

Immunostaining for vinculin and paxillin showed characteristic focal streaks in cells plated on control collagen but these structures were barely visible in cells plated on 0.1 mM or 1 mM MGO-treated collagen (Fig. 8A,B). Quantification of the projected area of these structures by morphometric analysis indicated that MGO treatment of collagen substantially reduced the abundance of paxillin and vinculin-stained focal adhesions in cells ($p < 0.01$; Fig. 8C).

3.7. Intercellular adhesions

The lack of effect of β 1 integrin activation on SMA expression, the inhibition of focal adhesion formation by MGO-collagen and the requirement for β 1 integrin interactions with collagen indicated that other adhesion receptors may cooperate with β 1 integrins to mediate SMA expression in human cardiac fibroblasts. Accordingly, we examined the effect of plating cells on MGO-collagen using antibodies against OB-cadherin, pan-cadherin and β -catenin, which are expressed by fibroblasts undergoing differentiation to myofibroblasts (Hinz et al., 2004; Pittet et al., 2008). MGO-collagen strongly increased the density of immunoblots prepared with pan-cadherin and β -catenin antibodies (Fig. 9A) after 2 days of plating but we were unable to detect reactivity with OB-cadherin antibody in these cells. This was not because of technical limitations since lysates of osteoblastic cells (SAOS2) showed strong immunoreactivity with the antibody to OB-cadherin. Confocal immunofluorescence analysis of cells also showed that staining with the pan-cadherin antibody was detected in cells plated on MGO-collagen but not on control collagen (Fig. 9B). Staining with the pan-cadherin antibody disappeared when cells plated on MGO-collagen that had been co-incubated with aminoguanidine (1 mM MGO overnight treatment of collagen fibrils attached to plates and co-incubation with 20 mM aminoguanidine to prevent the formation of glycosylated proteins). Further, if cells were plated at lower densities (i.e. 3.0×10^4 cells per 28 mm^2 of collagen substrate instead of the usual 6.0×10^4 cells per 28 mm^2 of collagen substrate) staining with the pan-cadherin antibody was

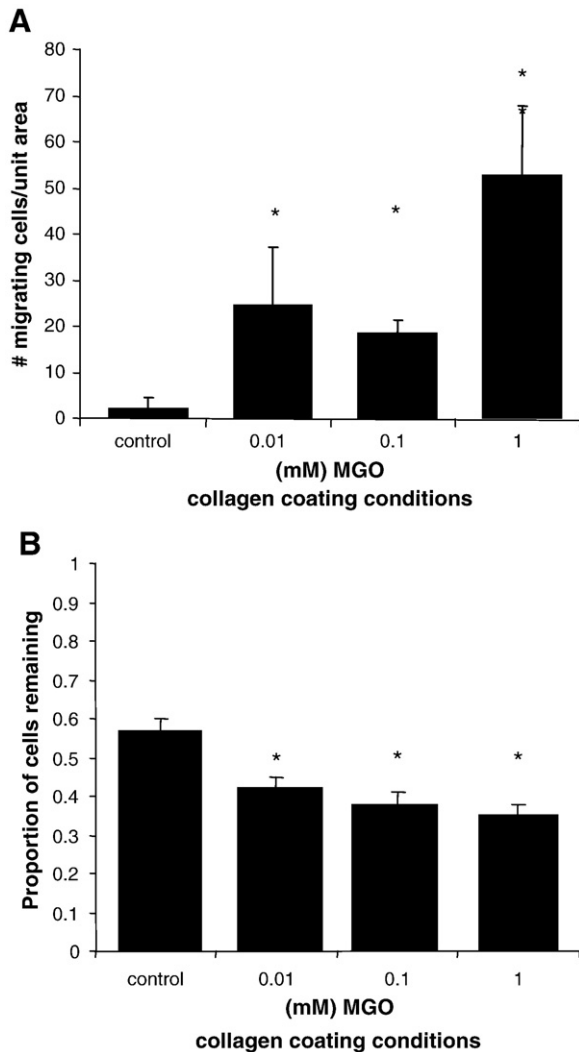


Fig. 5. (A) Cell migration on collagen or MGO-collagen examined using Transwell assays. Data are mean \pm standard error of mean number of cells that had migrated on to lower side of membrane. Each unit area was a 250 \times power microscope field (4 fields per membrane; 4 separate cultures). * indicates $p < 0.01$ and ** indicates $p < 0.001$ different than collagen controls. (B) Adhesion after application of shear force. Data are mean \pm standard error of mean proportion of cells remaining per mm^2 of micro-channel surface for different indicated coating conditions. Data are plotted against shear levels. Cells plated on collagen remain attached more tightly than cells plated on MGO-collagen ($p < 0.05$ for control collagen versus 1 and 10 mM MGO).

undetectable (Fig. 9B, right panel). By immunoblotting there were 1.85-fold higher ratios of SMA/GAPDH for cells on 6.0×10^4 cells per 28 mm^2 of collagen substrate compared to cells plated at 3.0×10^4 cells per 28 mm^2 of collagen substrate.

Plating cells on MGO-collagen also induced the expression of another protein that is associated with myofibroblast differentiation, ED-A fibronectin (Fig. 9C). The expression of ED-A fibronectin, cadherin and SMA was not inhibited if cells plated on MGO-collagen were co-incubated with 10 μM Y27632, an inhibitor of the Rho kinase.

As our quantitative RT-PCR data showed no detectable TGF- β 1, a reduction of TGF- β 3 and a 5-fold increase of TGF- β 2 after 2 days of culture (Fig. 4D), we determined if absorption of all TGF- β isoforms with an inhibitory antibody may affect MGO-collagen-induced SMA expression. Cells were plated on MGO-collagen or control collagen and co-incubated with TGF- β neutralizing antibody (50 $\mu\text{g}/\text{ml}$, changed daily for 3 days (Walker et al., 2004)). The MGO-collagen-induced increase of SMA was blocked compared to controls (Fig. 9D; $p > 0.2$).

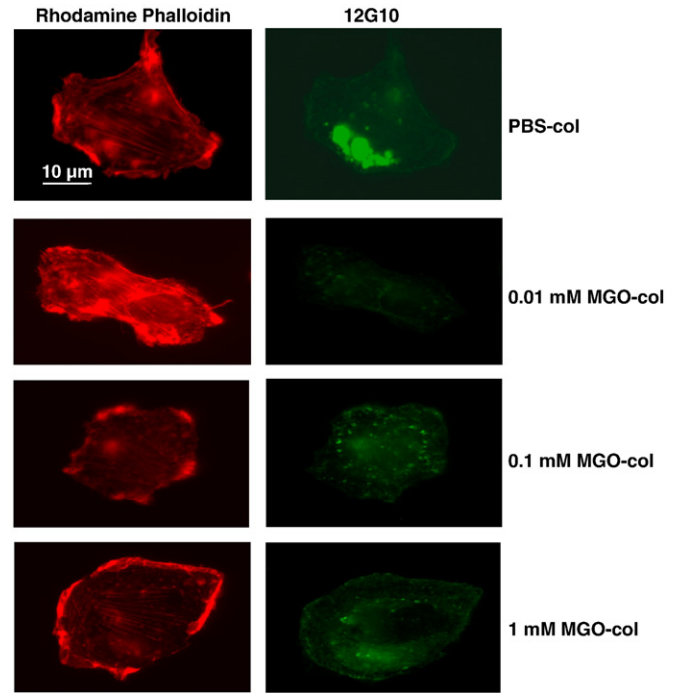


Fig. 6. Fluorescence micrographs of rhodamine phalloidin staining for actin filaments and for activated, ligand binding-induced neo-epitope on β 1 integrin subunits using the 12G10 antibody. Integrin activation is clearly seen only in cells plated on control collagen. Note that rhodamine actin staining in cortical regions is not markedly affected by MGO-collagen.

4. Discussion

Despite the greatly increased prevalence of heart failure in diabetics (Kannel et al., 1974) and the well-defined role of the myofibroblast in the formation of fibrotic lesions in the heart (Brown et al., 2005; Baudino et al., 2006; Campbell and Katwa, 1997), the impact of diabetes-induced glycation of extracellular matrix proteins in myofibroblast formation has not been defined. Our central findings indicated that treatment of collagen with the diabetic metabolite MGO enhances the differentiation, survival and migration of myofibroblasts, which in turn facilitates collagen matrix contraction. These results arise from the inhibitory effect of MGO-induced glycation on the formation of high affinity β 1 integrin-dependent adhesions and point to a central role for diabetic metabolites in regulating the migratory behavior, selection and persistence of myofibroblasts in the cardiac interstitium of diabetics.

4.1. Integrins and collagen glycation

Integrin-dependent interactions with matrix proteins mediate cell polarization, extension of lamellipodia and filopodia, formation and stabilization of attachments, and generation of tractional forces (Sheetz, 1994) and cell migration (Lauffenburger and Horwitz, 1996). In migrating cells β 1 integrins are concentrated in contacts at the leading edge (Burridge et al., 1988). For migration and remodeling

Table 1

Floating collagen gel contraction assays (slopes of best fit linear curves). Data were analyzed by analysis of variance. $N = 4$ replicates per condition. * $p < 0.05$ from integrin, PBS control. ** $p < 0.01$ from integrin, PBS control.

Conditions	PBS control	0.1 mM MGO	1 mM MGO
Integrin control	4.5 \pm 0.07	4.6 \pm 0.09	6.1 \pm 0.10*
Integrin activator	4.9 \pm 0.13	6.07 \pm 0.12*	6.2 \pm 0.18*
Integrin inhibitor	0.81 \pm 0.06**	0.77 \pm 0.07**	1.0 \pm 0.22**

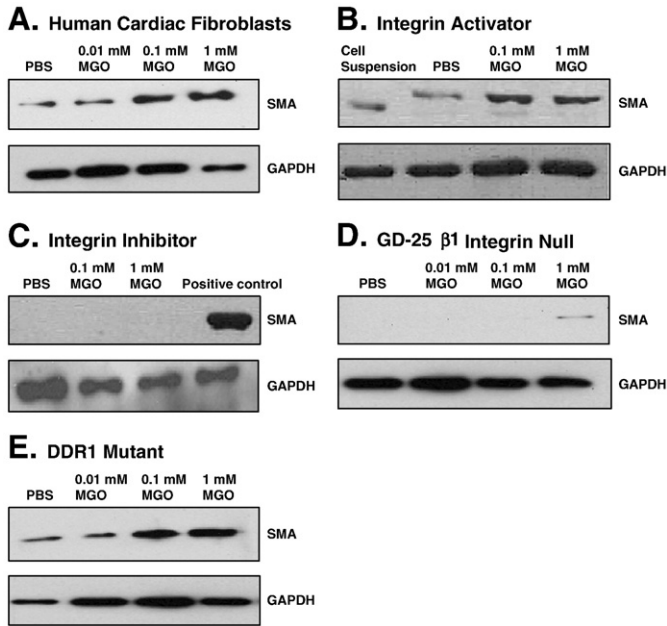


Fig. 7. Immunoblotting for SMA and GAPDH in human cardiac fibroblasts (A–C), GD-25 cells and DDR1 null cells as indicated. Cells were cultured on control collagen, MGO-collagen or in suspension. (A) Cells on MGO-collagen express higher levels of SMA than control collagen. (B) Treatment with activating antibody (12G10) does not substantially alter SMA expression compared to cells in A. (C) Cells incubated with β 1 integrin-inhibiting antibody (4B4) show no SMA expression in spite of plating on MGO-collagen. (D) GD-25 cells, which are null for β 1 integrin, also did not express SMA. (E) DDR-1 null cells show similar levels of SMA as HCF cells plated on MGO-collagen.

by fibroblasts on type I collagen fibrillar matrices, the principal integrin is the α 2 β 1 (Klein et al., 1991). We found by immunostaining that MGO-collagen inhibits the formation of high affinity β 1 integrin adhesions, as detected with an activation-dependent, neo-epitope antibody. Consistent with these data, shear adhesion assays showed decreased adhesion strength to MGO-collagen. MGO interacts with lysine, cysteine and arginine residues to form glycation adducts that perturb β 1 integrin–collagen interactions (Paul and Bailey, 1999). In particular, MGO preferentially reacts with arginine to form imidazolones, advanced glycation end products that exhibit loss of associated side-chain positive charge (Ahmed et al., 2002). Notably, the α 2 β 1 integrin recognizes and binds the GFOGER sequence in fibrillar collagens (Emsley et al., 2000). MGO adduct formation with the arginine in the GFOGER sequence of collagen appears to be particularly important in modifying and inhibiting β 1 integrin-dependent cell attachment and spreading on collagen (Chong et al., 2007), processes that may be critical for inhibition of high affinity adhesions as observed here. Recent reports have shown that methylglyoxal modifications have important effects on cell adhesion-related phenomena including proliferation, apoptosis and migration (Dobler et al., 2006; Pedchenko et al., 2005; Pozzi et al., 2009).

4.2. Cell migration

Initial formation of cell adhesions at the leading edge of migrating cells is marked by the involvement of actin binding proteins such as vinculin, a regulator of cell migration and adhesion (DePasquale and Izzard, 1987; Zimmerman et al., 2004). After vinculin recruitment to cell adhesions the cell-to-matrix distance is reduced, which leads to the formation of higher affinity (DePasquale and Izzard, 1987) and stable adhesions (Geiger and Bershadsky, 2001). The anchorage provided by these adhesions facilitates transmission of cell-generated forces to enable migration (Lauffenburger and Horwitz, 1996) but if the cells are very highly adherent to the matrix, migration is impeded. We found that the relative abundance of vinculin and paxillin in cell

adhesions was markedly reduced in cells plated on MGO-collagen. These results are consistent with findings that show vinculin null cells form few focal adhesions and do not spread well, but migrate more rapidly (Rodriguez Fernandez et al., 1992). Accordingly, our data on MGO-collagen enhancement of cell migration in Boyden chamber assays may be explained by the inability of cells to form high affinity focal adhesions on glycated collagen.

4.3. Collagen remodeling

After prolonged hyperglycemia, the extracellular matrix of the cardiac interstitium can be profoundly affected in diabetic patients, which may be manifest as increased cross-linking of collagen and alteration of its functional properties. We found that for both stress-relaxed and floating gels (Grinnell, 2000) collagen glycation by MGO enhanced gel contraction. Two proposed mechanisms may account for cell-mediated contraction and re-organization of collagen matrices. The first mechanism suggests that isometric tension applied to collagen fibrils by cells results in gel contraction (Dodd et al., 1982), which is in agreement with our observations that MGO-collagen enhanced SMA expression, and that SMA content of fibroblasts increases collagen gel contraction (Arora and McCulloch, 1994). Notably, increased SMA expression is strongly associated with enhanced contractility (Arora and McCulloch, 1994; Tomasek et al., 2002). The second mechanism suggests that tractional forces arising from the combined motility and contractility of cells exert shear forces tangential to their surface (Harris et al., 1981). Since we also observed that MGO treatment increased cell migration on collagen, at least part of the more rapid collagen gel contraction can be attributed to migration through the gels (Harris et al., 1981; Roy et al., 1999) while for the stress-relaxed collagen gels, which depend on factors that affect cell contractility (Tarpila et al., 1998), SMA expression may be more important.

4.4. SMA expression

The formation of myofibroblasts is a critical process in heart failure since myofibroblasts produce a densely fibrotic extracellular matrix that reduces cardiac contractility and inhibits filling in diastole (Weber et al., 1997). Myofibroblasts are characterized by *de novo* expression of collagen, SMA and, under certain conditions, ED-A fibronectin, as well as the formation of actin stress fibers *in vitro* (Tomasek et al., 2002). We found that SMA and ED-A fibronectin expression in human cardiac fibroblasts was enhanced by MGO treatment of collagen, in spite of the reduced adhesion strength to the glycated extracellular matrix. The MGO-induced glycation of collagen was evidently a key factor in this conversion process since co-incubation with aminoguanidine (Lo et al., 1994) blocked MGO-induced glycation of collagen and SMA expression.

When cultured on MGO-collagen substrates, SMA was found in well-developed actin bundles that appeared to terminate in adhesions proximal to adjacent cells, suggesting that some type of intercellular adhesion was involved in providing the anchorage point for the stress fibers. Previous reports have indicated that N- and OB-cadherins are important for myofibroblast differentiation (Hinz et al., 2004; Pittet et al., 2008) and our data show that the increased expression of cadherins (possibly by P-cadherin as detected by RT-PCR but not OB-cadherin) and β -catenin, important intercellular adhesion molecules, may be involved in enhanced myofibroblast differentiation in response to MGO-collagen. If cells were cultured at lower densities, which reduced the likelihood of intercellular adhesion contacts between adjacent cells, the MGO-collagen-induced expression of SMA dissipated. The induction of SMA expression by plating cells on MGO-collagen evidently does not involve the Rho kinase pathway since incubation of cells on MGO-collagen and treatment with Y27632 did not affect SMA expression, indicating that cell-generated tension

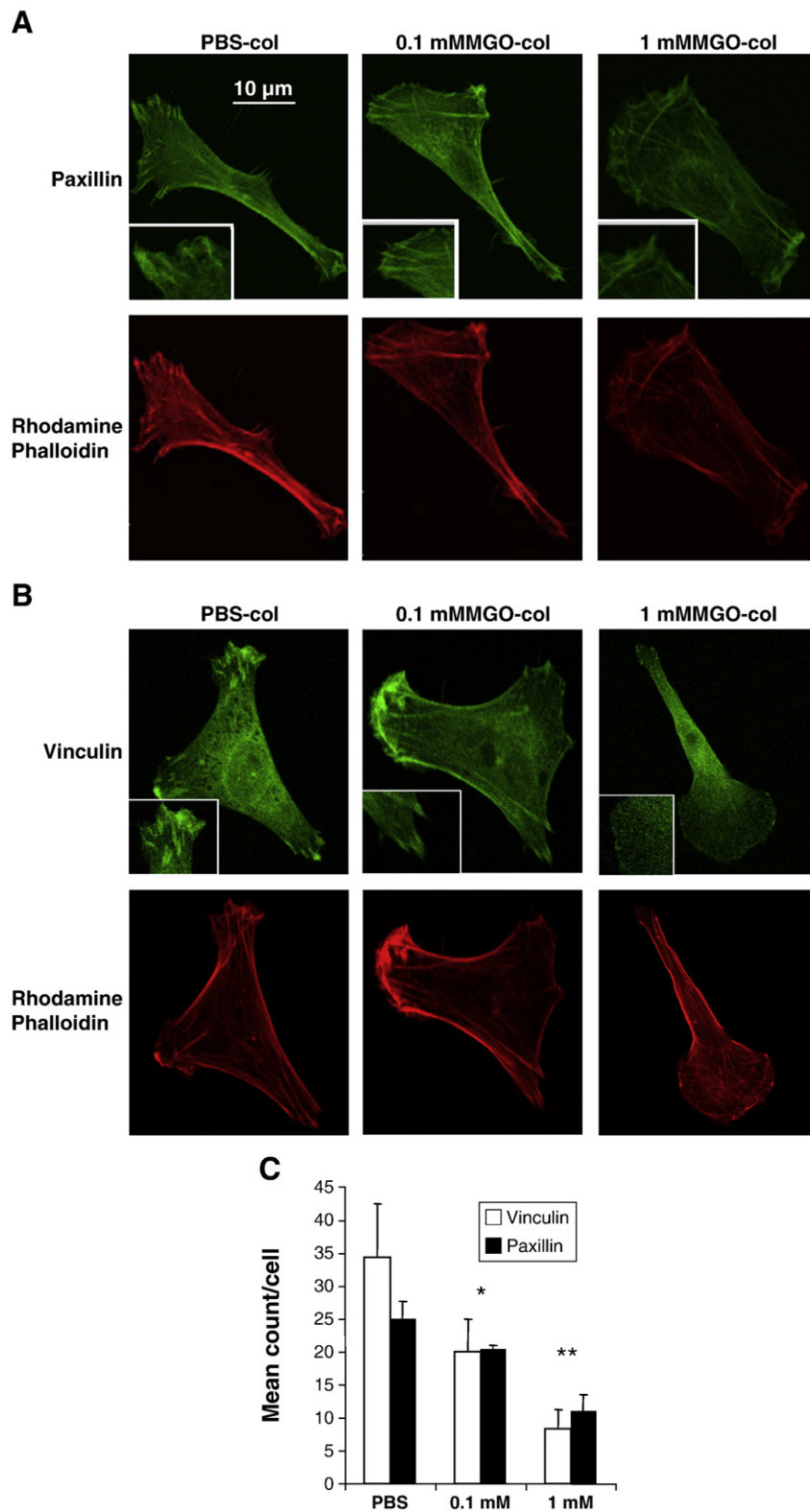


Fig. 8. (A,B) Fluorescence micrographs of immunostaining for paxillin and vinculin. Cells were counter-stained with rhodamine phalloidin for actin filaments in human cardiac fibroblasts 1 day after culture on slides coated with control collagen or 0.1 mM or 1 mM MGO-collagen. Plating on MGO-collagen inhibited formation of paxillin and vinculin-stained adhesions. (C) Morphometric analysis of number of vinculin or paxillin-stained focal adhesions per cell. Data are mean \pm standard error of number of focal adhesions counted in 10 cells from 4 different cultures. * indicates $p < 0.05$ and ** indicates $p < 0.01$ different than collagen controls.

(Tomasek et al., 2002) may not be an important factor in mediating this process.

The MGO-collagen induction of SMA expression involved selectively increased expression of TGF- β 2 and required TGF- β since

blockade of this cytokine with neutralizing antibodies blocked MGO-collagen-induced enhancement of SMA expression. TGF- β neutralizing antibodies have been used earlier in studies of the differentiation of heart valve myofibroblasts (Walker et al., 2004). Further, SMA

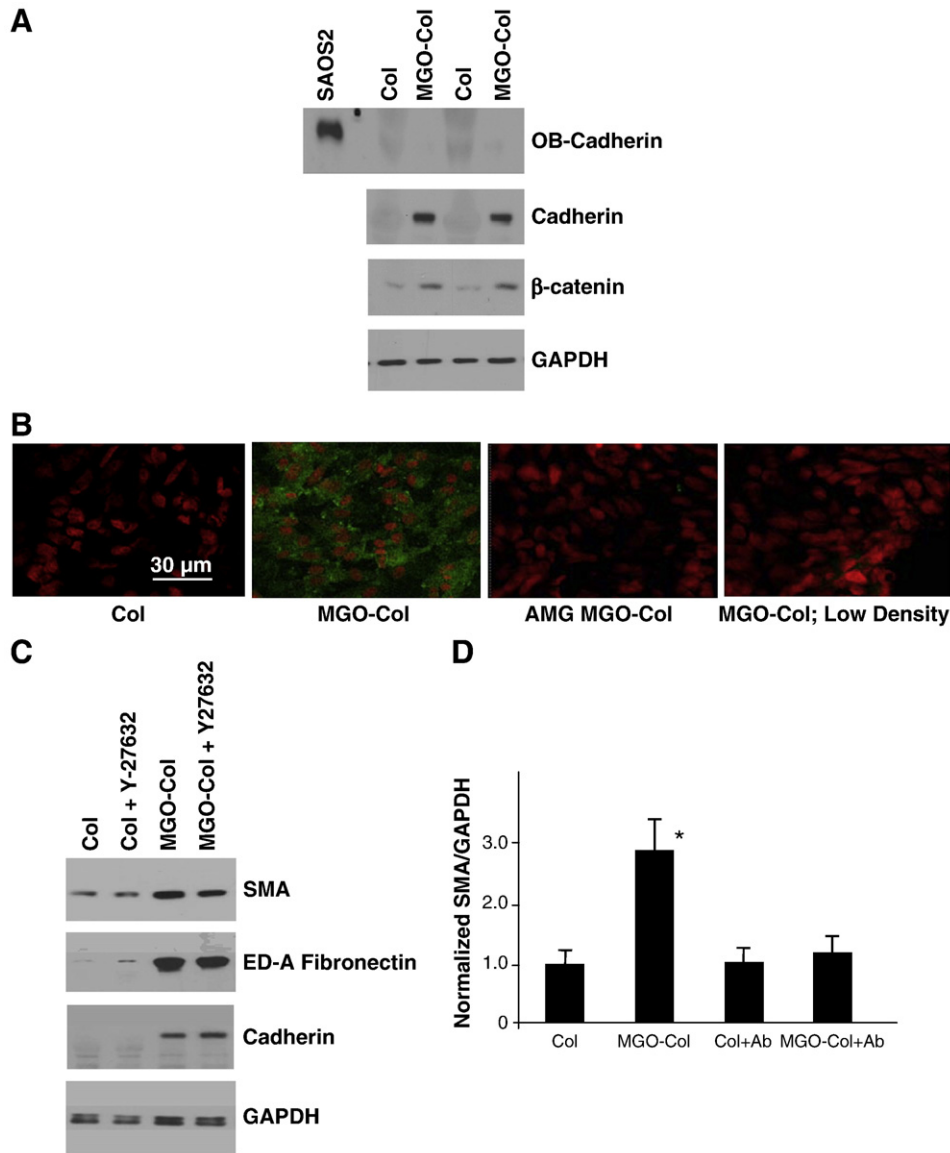


Fig. 9. (A) Immunoblotting of human cardiac fibroblasts plated on control or 1 mM MGO-collagen for indicated intercellular adhesion proteins. SAOS2 cell lysates were used as a positive control for OB-cadherin. (B) Human cardiac fibroblasts were plated at high density on control collagen (Col), 1 mM MGO-collagen (MGO-Col), or co-incubated with 20 mM aminoguanidine and 1 mM MGO-collagen (AMG MGO-Col) or plated at low density on 1 mM MGO-collagen (MGO-Col; Low Density). Cells were immunostained with antibody to pan-cadherin and counter-stained with propidium iodide to visualize nuclei. Note that cadherin staining fills the cytoplasm of cells on MGO-collagen and extends to the adjacent cell membrane. (C) Human cardiac fibroblasts were plated on control collagen or 1 mM MGO-collagen in the presence or absence of 10 μ M Y27632, an inhibitor of the Rho kinase. Cells were immunoblotted for the indicated myofibroblast markers. (D) Ratios of mean immunoblot densities of SMA and GAPDH normalized to control cell values of 1.0 in cells treated as indicated. Data are normalized densities \pm standard errors of the mean. Data are for cells plated on collagen (Col), or on MGO-collagen (MGO-Col), or cells pre-incubated with a TGF- β neutralizing antibody on collagen (Col + Ab) or cells pre-incubated with the neutralizing antibody and plated on MGO-collagen (MGO-Col + Ab).

expression in high density cultures was strongly linked to survival from MGO-collagen-induced apoptosis, consistent with the notion that intercellular adhesions, possibly mediated by cadherins and β -catenin, can enable the persistence of myofibroblasts when cultured on MGO-collagen. Indeed, the selective inhibition of apoptosis in SMA-expressing myofibroblasts by MGO-collagen may suggest a mechanism by which these cells persist in glycosylated extracellular matrices.

Stress fiber formation, cadherins, SMA, and ED-A fibronectin expression are considered to be important markers for myofibroblasts (Hinz et al., 2004; Tomasek et al., 2002). In addition to the apparent role for intercellular adhesions in myofibroblast formation, our data on the role of β 1 integrins in this process also show that attachment to collagen is important for enhanced SMA expression. Otherwise, if β 1 integrin-collagen interactions were inhibited (by the 4B4 antibody),

then SMA expression was blocked. Hinz et al. (2004) have suggested a model for myofibroblast formation in which some cells in the fibroblast population attach to the extracellular matrix while other cells attach strongly to each other by intercellular adhesions. They consider that the formation of fibrogenic fibroblasts requires the formation of intercellular adhesions to tether and provide critical signals for myofibroblast formation (Follonier et al., 2008). These fibrogenic cells, which express SMA and ED-A fibronectin, evidently do not need to be tightly adherent to the extracellular matrix, which might explain our results indicating that inhibition of the Rho kinase does not interfere with myofibroblast formation and that MGO-collagen is associated with reduced integrin adhesion. Taken together, our data indicate that glycosylated collagen inhibits the formation of high affinity, β 1 integrin-dependent adhesions to the extracellular matrix

while facilitating intercellular adhesion formation and cell migration over collagen, processes which are important for the generation of myofibroblasts and their spread through the diabetic myocardium.

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Author contributions: AY, CAS, and CAM conceived and designed the experiments. AY, CL, IT, WL, MC, JB, EWKY, KS, and EW performed the experiments. AY, CAS, and CAM analyzed the data. AY, CL, CAS, and CAM wrote the paper.

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