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14. ABSTRACT

Scleroderma is a disease where excess collagen is deposited in the skin and internal organs. The tissues become hard and in the end fail to function. To date there is no cure, nor, is there an effective therapy that will control the deposition of the collagen. The goals of this application were to investigate the cellular signaling within fibroblasts that were mediated by the glycation end product, 3DG. We find that 3DG decreases the expression of collagens and therefore we proposed to understand the cellular signaling in fibroblasts in response to this compound. Specifically we found decreased expression of ERK1/2 and MEK1/2 phosphorylation, reduction on collagen specific transcription factors, increased adherence to the 3DG-collagen and that α1β1 integrin is the most important integrin for binding 3DG-collagen. We have found further perturbations in fibroblast signaling with 3DG-collagen, including increased GADD153 expression, p38 MAP kinase and Smad7. These alterations contribute to the decreased expression of collagen. We continue to further unravel the alterations observed in signaling with 3DG.

15. SUBJECT TERMS
3-deoxyglucosone (3DG), scleroderma (SSc), caspase, apoptosis, collagen, integrin, extracellular matrix, fibroblasts

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INTRODUCTION

The goals of this application were to investigate the cellular signaling within fibroblasts that were mediated by the glycation end product 3-deoxyglucosone (3DG). In the preliminary data in the grant, we demonstrated that collagen expression and TGF-β was decreased in fibroblasts cultured on 3DG-modified collagen matrices. We stated in the abstract the following; "this grant seeks to better understand the altered signaling between the extracellular matrix and fibroblasts isolated from the fibrotic lesions from systemic sclerosis (SSc) patients". The goals of the experiments proposed are a natural progression of the provocative preliminary data and will investigate the mechanism as to how 3DG can modify the signaling from the extracellular matrix via integrins, signaling through the ERK pathway. **Specifically, we propose that fibroblasts respond differentially to extracellular matrix that has been modified by 3DG. We hypothesize that this modification causes a feedback signal through the ERK pathway into the fibroblasts that results in the altered expression of pertinent transcription factors that in turn affect COL1A1, COL3A1, elastin, fibrillin-1, connective tissue growth factor, and TGF-β gene expression.** More importantly, we believe that this mechanism can be utilized to modulate the fibrotic events observed in scleroderma.

BODY

We have now received all 16 cell lines that we estimated we require for the experiments proposed from Carol Feghali-Bostwick, PhD at Pittsburgh University. We have also purchased a total of 16 aged matched normal cell lines from the Coriell Institute (Camden NJ) with which the comparisons were made, as we previously determined that it is important to have aged matched controls in these experiments as collagen expression decreases with increasing age (see Figure 1 of the 2007 – 2008 Annual Report). The goals of this application were to elucidate the changes in signaling in fibroblasts cultured on 3DG-collagen so that we can identify a suitable inhibitor that can be used therapeutically to treat SSc.

Therefore, we have further elucidated the signaling induced by 3DG-collagen by investigating endoplasmic reticulum (ER) stress responses and we have found that in the presence of 3DG-collagen, p38 MAPK switches from a growth kinase to a stress kinase.

1. ER stress mediates 3DG-collagen-induced caspase-3 activation in human dermal fibroblasts

We found that 3DG-collagen induced the apoptotic signaling cascade in human dermal fibroblasts; therefore we wanted to determine if this was dependent on ER stress. Fibroblasts were cultured on native collagen or 3DG-collagen coated dishes with or without aminoguanidine (AG-inactivator of 3DG), or treated with or without meglumine (3DG inhibitor, termed Dyn15 in the grant application) for 24 h. Apoptosis was measured by the expression of active caspase-3. Fibroblasts cultured on 3DG-collagen induced a 150% $\pm$ 4.5% increase in active caspase-3 expression compared to fibroblasts grown on native collagen (Figure 1, $p < 0.001$). When fibroblasts were treated with AG the activity of caspase-3 was reduced to levels observed in fibroblasts grown on native collagen. Additionally, meglumine, prevented the 3DG-collagen-induced increase in caspase-3 activity (Figure 1).

ER stress induces apoptosis of the cell (1-4). Therefore, fibroblasts were pretreated with the ER stress inhibitor salubrinal and cultured on native collagen or 3DG-collagen for 24 h. Treatment with salubrinal reduced the level of active caspase-3 within fibroblasts cultured on 3DG-collagen to that of fibroblasts cultured on native collagen (Figure 1, $p<0.0002$). These results suggest that 3DG-collagen can induce the activation of the apoptotic signaling cascade through ER stress. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

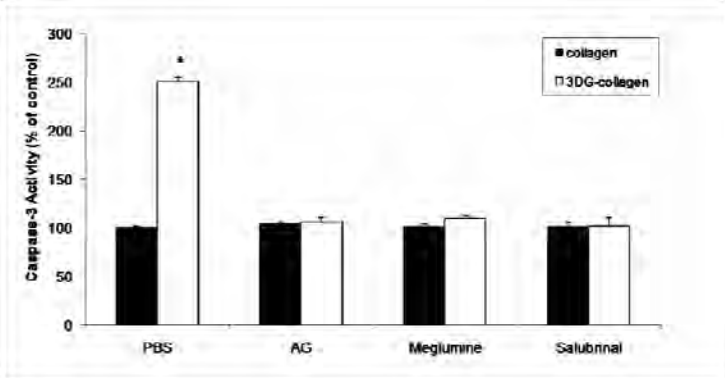


Figure 1. Induction of caspase-3 activity by 3DG-collagen is dependent on ER stress. Fibroblasts were cultured on native collagen or 3DG-collagen and treated with or without 5 mM AG, 40 mM meglumine, or 40 µM salubrinal for 24 h. 100 µg of whole cell lysate was assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against collagen treated with PBS. Data are mean $\pm$ SD (n=3), *P < 0.0002.

2. ER stress-induced apoptosis marker GADD153 is upregulated in fibroblasts cultured on 3DG-collagen

Accumulation of misfolded proteins within the ER can lead to stress and induction of GADD153, a transcription factor involved in apoptosis (1-4). We found that GADD153 transcript levels and activation of GADD153 in fibroblasts by 3DG-collagen was increased (6). Moreover, meglumine was found to inhibit 3DG-collagen-induced GADD153 expression (6). To further confirm that 3DG-collagen is inducing ER stress, fibroblasts were pretreated with salubrinal, an inhibitor of ER stress, and cultured on 3DG-collagen for 24 h. The fibroblasts were then stained for GADD153 and inspected for GADD153 localization within the nucleus, which is indicative of activated GADD153 (7). Mean fluorescent intensity (MFI) of the nuclei was measured. In the presence of salubrinal, fibroblasts cultured on 3DG-collagen reduced the expression of GADD153 to that observed in fibroblasts cultured on native collagen (11.2 MFI $\pm$ 2.0 with salubrinal compared to 30.5 MFI $\pm$ 2.1 on 3DG-collagen), further confirming that 3DG-collagen is inducing ER stress in dermal fibroblasts (Figure 2A, $p < 0.007$). To verify the immunofluorescence results, a Western blot was performed. Confirming the immunofluorescence observations, Western blot analysis demonstrated an increase to 203% $\pm$ 4.1% in GADD153 expression in fibroblasts cultured on 3DG-collagen compared to fibroblasts cultured on native collagen ($p<0.0005$). AG and meglumine reduced the level of GADD153 expression in fibroblasts cultured on 3DG-collagen to 91% $\pm$ 5.2% ($p<0.0005$) and 92% $\pm$ 4.2% ($p<0.0005$), respectively and salubrinal prevented 3DG-collagen-induced increase of GADD153 ($p<0.0005$) (Figure 2B). These findings suggest that 3DG-collagen is inducing apoptosis through the ER stress-signaling pathway, which is dependent on GADD153 activation. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

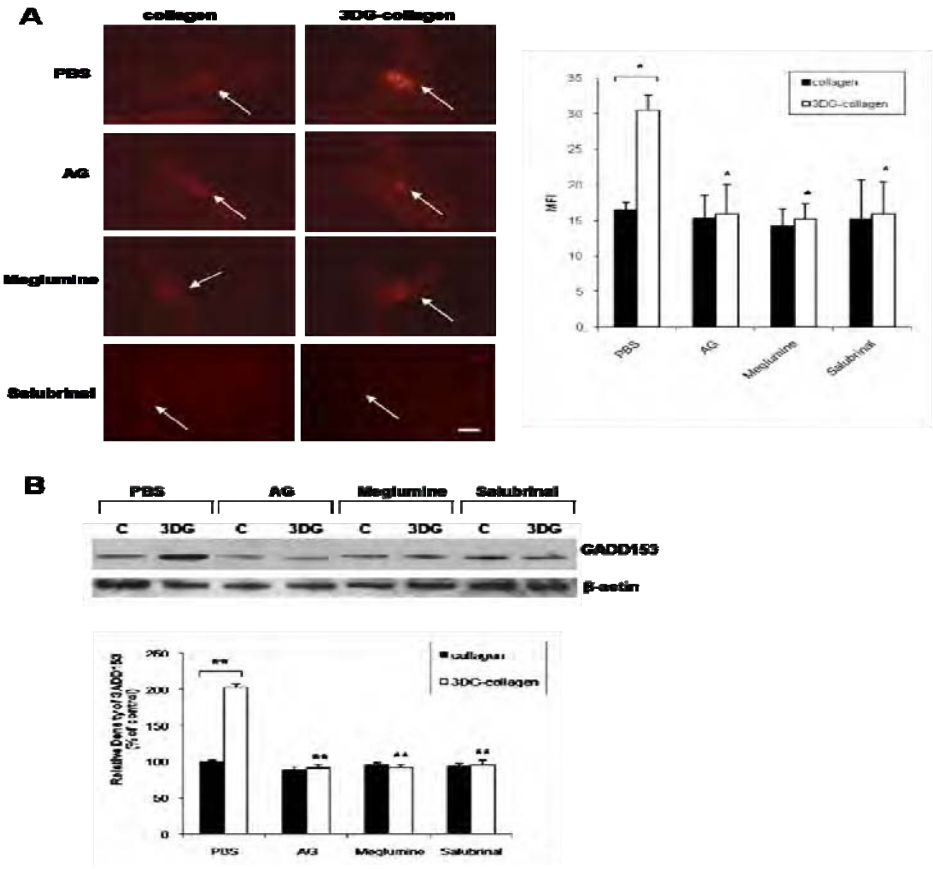


Figure 2. Effect of ER stress inhibitor salubrinal on 3DG-collagen-induced GADD153 expression. A, Fibroblasts were cultured in chamber slides coated with native collagen or 1 mM 3DG-collagen with or without 5 mM AG or 40 mM meglumine for 24 h. Also, fibroblasts were pretreated for 1 h with or without 40 µM salubrinal and then cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts were stained and analyzed for expression of GADD153 in the nucleus by immunofluorescence analysis using Cy3-conjugated secondary antibody. Mean fluorescence intensity (MFI) of GADD153 in the nucleus was measured using ImageJ from ten representative fibroblasts. Images were taken at 40 X magnification on an epi-fluorescent microscope. Arrows indicate nuclei containing GADD153. The bars represent the MFI values from each experimental condition. Scale bar represents 10µm. **B**, Fibroblasts were treated as in **A** followed by Western blot for GADD153 expression. β-actin was used as a loading control. The bars represent the densitometric value for each experimental condition. All comparisons are made against 3DG-collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), **P < 0.0005, *P < 0.007.

3. 3DG-collagen stimulates ROS in dermal fibroblasts

ROS are known to cause oxidative stress and have been linked to the activation of GADD153-induced apoptosis in cells (4;8;9). Therefore, we determined if ROS was produced during the culturing of fibroblasts on 3DG-collagen. Fibroblasts cultured on 3DG-collagen produced 376 nM ± 3.4 of intracellular ROS at 24 h in comparison to the 38.7 nM ± 2.2 of ROS produced by fibroblasts grown on native collagen (Figure 3, $p<0.001$). This increase was comparable to that observed with hydrogen peroxide (H_2O_2), a free radical involved in ER stress, which produced 458 nM ± 3.2 (Figure 3, $p<0.001$). Moreover, AG abrogated the rise in ROS only in fibroblasts cultured on 3DG-collagen, and not in cells treated with H_2O_2 suggesting that 3DG-collagen is specifically producing ROS (Figure 3; 94.3 nM ± 4.0 for 3DG-collagen/AG; 440.6 nM ± 4.0 for H_2O_2/AG, $p<0.001$). Meglumine inhibited the production of ROS in cells cultured on 3DG-collagen and partially inhibited ROS in cells treated with H_2O_2 suggesting that meglumine may prevent ROS induction by 3DG-collagen and/or may be a scavenger of free radicals (92.8 nM ± 4.0 for 3DG-collagen and meglumine; and 210.2 nM ± 4.4 for H_2O_2 and meglumine, $p<0.001$). In addition, the induction of ROS by fibroblasts cultured on 3DG-collagen, or treated with H_2O_2 could be blocked by pre-treating fibroblasts with the antioxidant ascorbic acid (Figure 3; 78.2 nM ± 3.6 for 3DG-collagen/ ascorbic acid; and 55.6 nM ± 4.8 for H_2O_2/ascorbic acid, $p<0.001$). Taken together, these results suggest that ROS could be produced in the fibroblast in response to the modification of collagen by 3DG. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

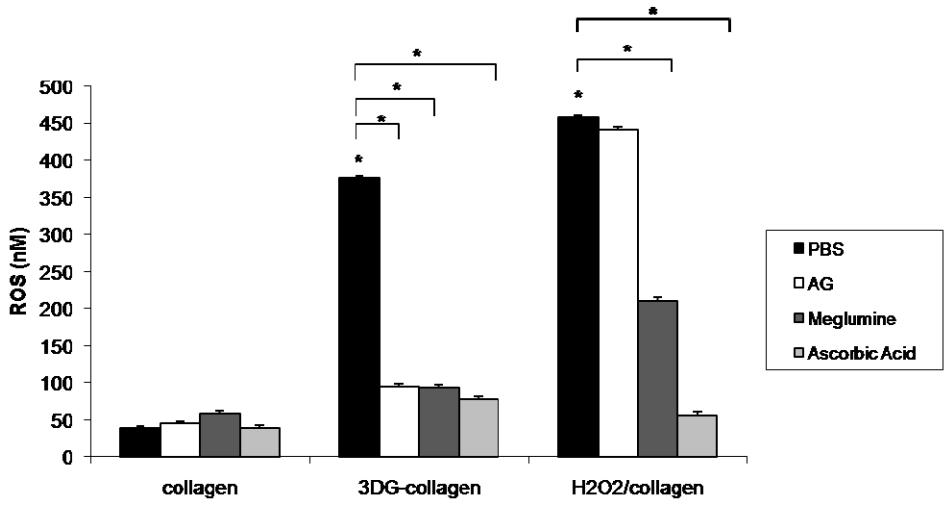


Figure 3. 3DG-collagen stimulates intracellular ROS in fibroblasts. Fibroblasts were cultured in 96-well plate coated with either native collagen or 1 mM 3DG-collagen or treated with or without 5 mM AG, 40 mM meglumine, or 100 µg/mL ascorbic acid for 24 h. Treatment of fibroblasts cultured on native collagen with 50 µM H_2O_2 was used as a positive control. Fibroblasts were loaded with DCFH-DA for 30 min and ROS production was measured by absorbance of fluorescent DCF at a wavelength of 480 nm/530 nm. Comparisons are made to collagen treated with PBS unless otherwise indicated. Data are mean $\pm$ SD (n=3), *P < 0.001.

4. NAD(P)H oxidase 4 (Nox4) is responsible for the 3DG-collagen-dependent production of ROS

The NAD(P)H oxidase controls the production of ROS through integrin activation, and cytokine and growth factor stimulation. Overexpression of key oxidases such as the non-phagocytic Nox4 has been associated with increased ROS and apoptosis (10-12). Nox4 has been shown to be highly expressed in fibroblasts compared to other NAD(P)H oxidase homologues (10;11). Therefore, we determined if 3DG-collagen-induced ROS were mediated by the overexpression of Nox4. Quantitative real-time PCR revealed that Nox4 mRNA expression increased to 880% $\pm$ 200.0% in fibroblasts cultured on 3DG-collagen for 24 h compared to fibroblasts cultured on native collagen (Figure 4A, $p<0.02$). Moreover, to ensure that Nox4 was the only Nox isoform being over-expressed by 3DG-collagen, quantitative real-time PCR was performed to determine the mRNA transcript levels of the other Nox isoforms, Nox1 and Nox2. Detection of Nox1 and Nox2 mRNA transcripts was not apparent suggesting that dermal fibroblasts over express specifically Nox4 (Figure 4A). To show specificity of 3DG, AG and meglumine reduced the transcript levels of Nox4 in fibroblasts cultured on 3DG-collagen to that observed in fibroblasts cultured on native collagen. Additionally, Nox4 protein levels were found to be increased in fibroblasts cultured on 3DG-collagen compared to fibroblasts cultured on native collagen (240% $\pm$ 8.6% in 3DG-collagen treated vs. 100% $\pm$ 3.3% in native collagen treated cells, Figure 4B, $p<0.001$). This upregulation was also abrogated by the 3DG inhibitors AG and meglumine. Immunofluorescence demonstrated increased Nox4 localization at the plasma membrane in the fibroblast when cultured on 3DG-collagen and that this increase was abrogated by AG and meglumine, suggesting that Nox4 may be activated on the cell surface (Figure 4C).

We further investigated the role of Nox4 in the upregulation of ROS in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with apocynin, a broad class NAD(P)H oxidase inhibitor, cultured on either native collagen, 3DG-collagen, or treated with H_2O_2 for 24 h, and intracellular ROS was quantified. Apocynin reduced ROS in fibroblasts cultured on 3DG-collagen to that observed in fibroblasts cultured on native collagen (Figure 4D, $p<0.001$). This further confirms that 3DG-collagen is inducing ROS through activation of Nox4. Furthermore, apocynin was found to only partially inhibit the level of ROS in fibroblasts cultured on native collagen with H_2O_2 suggesting that apocynin is inhibiting the ROS induced by the Nox4 complex rather than affecting the induction of ROS by exogenous H_2O_2 (Figure 4D, $p < 0.02$). This data was

published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

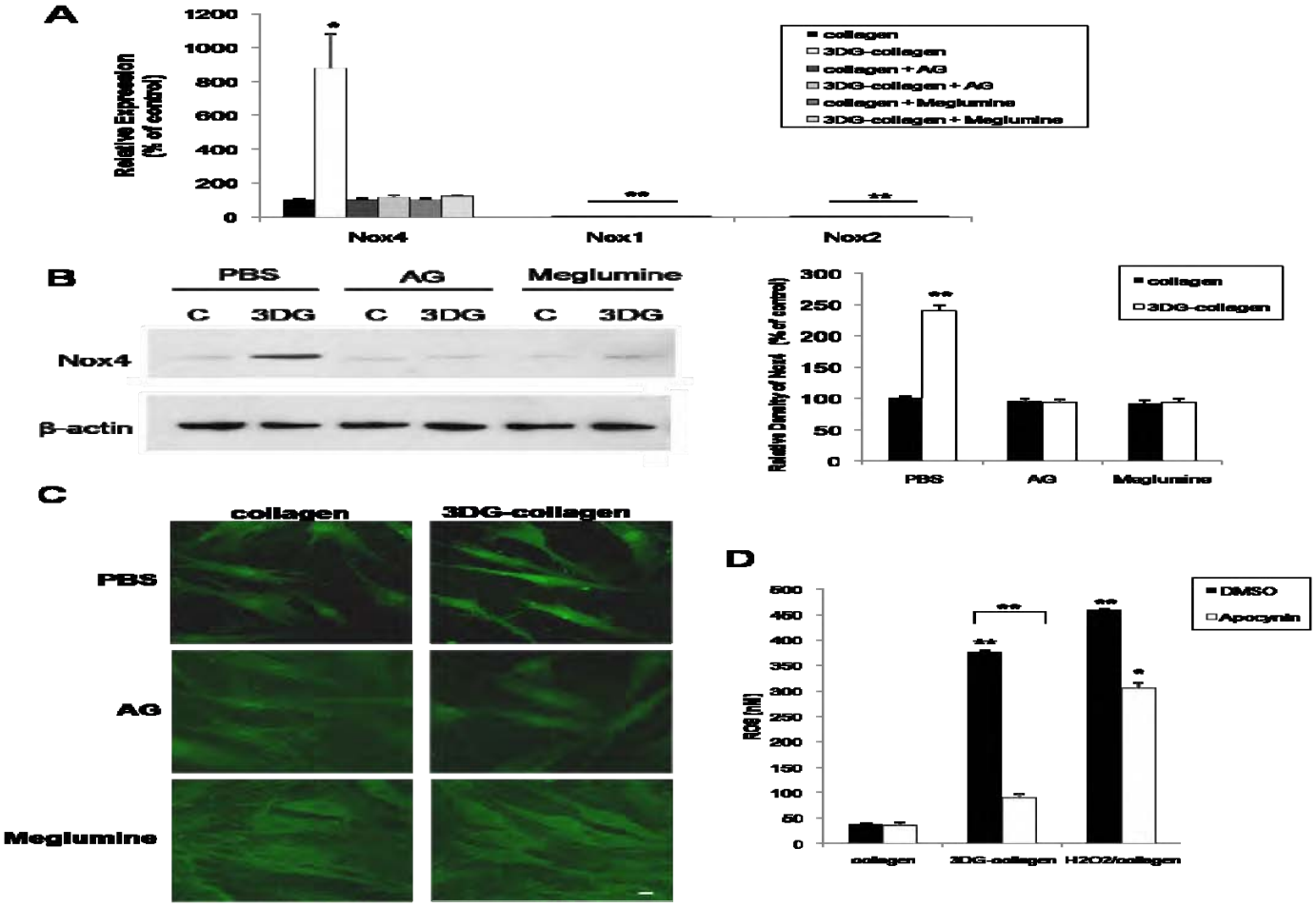


Figure 4. 3DG-collagen increases expression of Nox4 in the dermal fibroblast. Fibroblasts were cultured on either native collagen or 1 mM 3DG-collagen and treated with or without 5 mM AG or 40 mM meglumine for 24 h. **A**, Nox4, Nox1, and Nox2 mRNA expression levels were quantified by real-time PCR. All transcripts were normalized to β-actin. **B**, Expression levels of Nox4 were analyzed by Western blot and β-actin served as a loading control. Results were quantified by densitometric scanning of the Western blot and normalized for β-actin. **C**, Localization of Nox4 in fibroblasts treated the same as in **A** and **B** was analyzed by immunofluorescence with the anti-Nox4 polyclonal antibody and Cy2-conjugated secondary antibody. Images were taken at 40 X magnification on an epi-fluorescence microscope. Scale bar represents 10μm. **D**, Inhibition of Nox4 reduces the level of intracellular ROS. Fibroblasts were pretreated for 1 h with either vehicle, DMSO or NOX inhibitor, apocynin (1 mM) and cultured on native collagen, 1 mM 3DG-collagen, or cultured on native collagen and treated with 50 μM H_2O_2 for 24 h. Fibroblasts were then incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. Comparisons are made against collagen treated with DMSO and/or PBS unless otherwise indicated. Data are mean $\pm$ SD (n=3), **P < 0.001, *P < 0.02.

5. 3DG-collagen-induced phosphorylation of p38 MAPK is dependent on upstream ROS

During times of ER stress, ROS have been shown to activate the stress kinase p38 MAPK. To determine if 3DG-collagen-induced ROS are responsible for increased phosphorylation of p38 MAPK, fibroblasts were pretreated with the antioxidant ascorbic acid and the Nox inhibitor apocynin, and cultured on either native collagen, 3DG-collagen, or native collagen and treated with H_2O_2 and protein levels were measured by Western blotting. Fibroblasts cultured on 3DG-collagen increased the phosphorylation of p38 MAPK to 175% $\pm$ 4.1%. As a positive control for ROS-induced p38 MAPK activation, fibroblasts cultured on native collagen and treated with H_2O_2 increased the phosphorylated p38 MAPK to 181% $\pm$ 3.3%.

Pretreatment with ascorbic acid reduced the phosphorylation of p38 MAPK in fibroblasts cultured on 3DG-collagen or treated with H_2O_2 to that seen in fibroblasts cultured on native collagen (Figure 5, $p<0.0001$). Additionally pretreatment of with apocynin reduced the level of phosphorylated p38 to in fibroblasts cultured on 3DG-collagen, but not in fibroblasts treated with H_2O_2 (Figure 5, $p<0.0001$). These results suggest that 3DG-collagen-induced p38 MAPK is dependent on upstream production of ROS by Nox4. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

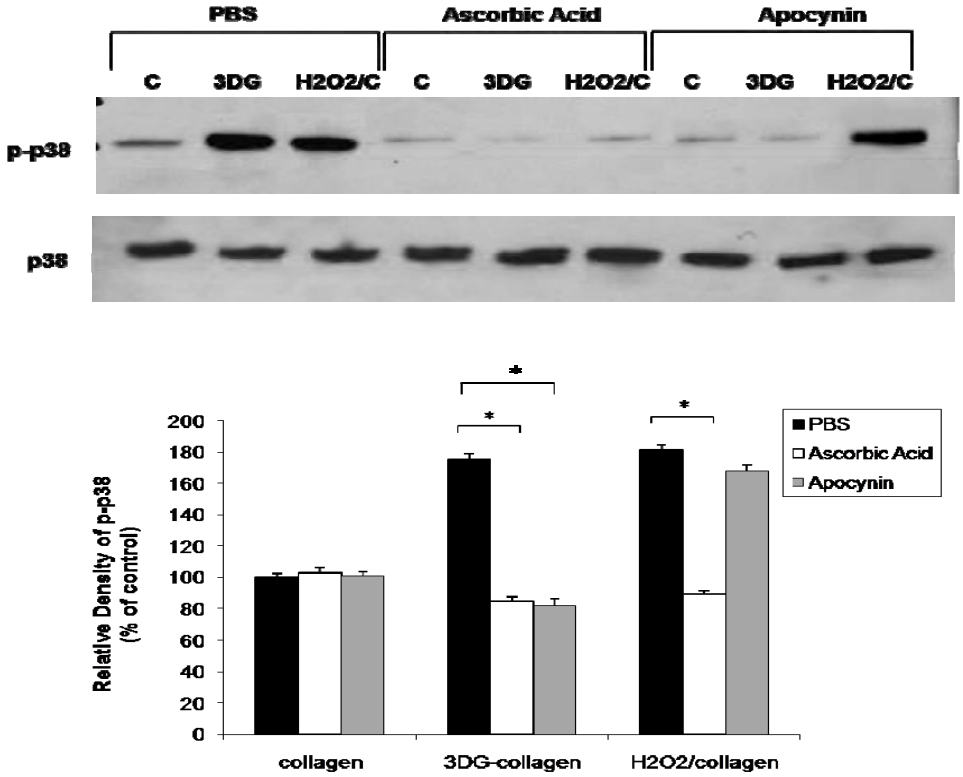


Figure 5. Phosphorylation of p38 MAPK is dependent on 3DG-collagen-induced ROS. Fibroblasts were pretreated for 1 h with 100 µg/mL of ascorbic acid, 1mM apocynin, or DMSO and cultured on either native collagen, 1 mM 3DG-collagen, or treated with 50 µM H_2O_2 for 24 h. Whole cell lysates were extracted and Western blot analysis of p-p38 MAPK was performed. Total p38 MAPK was used as a loading control. The bars correspond to the densitometric value of p-p38 MAP kinase after normalization for total p38 MAP kinase. Data are mean $\pm$ SD (n=3), *P < 0.0001.

6. 3DG-collagen-induced GADD153 expression is dependent on upstream ROS and p38 MAPK activation

3DG-collagen-induced ROS can lead to phosphorylation of p38 MAPK, which is essential for the activation of GADD153; therefore, the functional role of ROS and p38 MAPK in GADD153 induction was assessed (4;9;13). To determine whether GADD153 induction by 3DG-collagen was a result of free radical-mediated effects, fibroblasts were pretreated with ascorbic acid or apocynin and then cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts cultured on native collagen and treated with H_2O_2 were used as a positive control for ROS-induced GADD153 activation. The trafficking of GADD153 from the cytosol to the nucleus was found to be down regulated to 14.6 MFI $\pm$ 2.1 and 16.3 MFI $\pm$ 0.98 in response to ascorbic acid in fibroblasts cultured on 3DG-collagen or native collagen treated with H_2O_2, respectively (Figure 6A, $p< 0.007$). The expression of GADD153 in the nucleus of fibroblasts pretreated with apocynin and cultured on 3DG-collagen was also reduced to 13.9 MFI $\pm$ 1.2 (Figure 6A, $p< 0.007$). Western blot was performed to verify the expression of GADD153. GADD153 expression was decreased in response to ascorbic acid and apocynin in fibroblasts cultured on 3DG-collagen, while only ascorbic acid reduced the level of ROS in fibroblasts cultured on native collagen treated with H_2O_2 (Figure 6B; 81% $\pm$ 2.4% 3DG-collagen treated with ascorbic acid and 82% $\pm$ 4.6% treated with apocynin, and 75% $\pm$ 2.2% H_2O_2 treated with ascorbic acid and 188% $\pm$ 3.6% treated with apocynin, $p< 0.0001$). These results suggest that the generation of ROS by Nox4 lies upstream of GADD153.

Next, the role of p38 MAPK in GADD153 activation was assessed in fibroblasts cultured on native collagen or 3DG-collagen. Fibroblasts were pretreated with the p38 MAPK inhibitor SB202190 and cultured on native collagen, 3DG-collagen, or native collagen and treated with H_2O_2 for 24 h. Inhibition of p38 MAPK by SB202190 reduced the localization of GADD153 in the nucleus to 7.23MFI $\pm$ 1.13% in fibroblasts cultured on 3DG-collagen (Figure 6A, $p< 0.007$), and reduced the expression of GADD153 to 40% $\pm$ 4.0% (Figure 6A, $p<0.0001$). In addition, inhibition of p38 MAPK reduced the level of GADD153 expression in fibroblasts grown on native collagen treated with H_2O_2 to 51% $\pm$ 3.6% (Figure 6B, $p<0.0001$), and its nuclear localization to 8.21MFI $\pm$ 2.4 (Figure 6A, $p< 0.007$). These results suggest that the induction of p38 MAPK by upstream ROS is responsible for the activation of GADD153 by 3DG-collagen. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

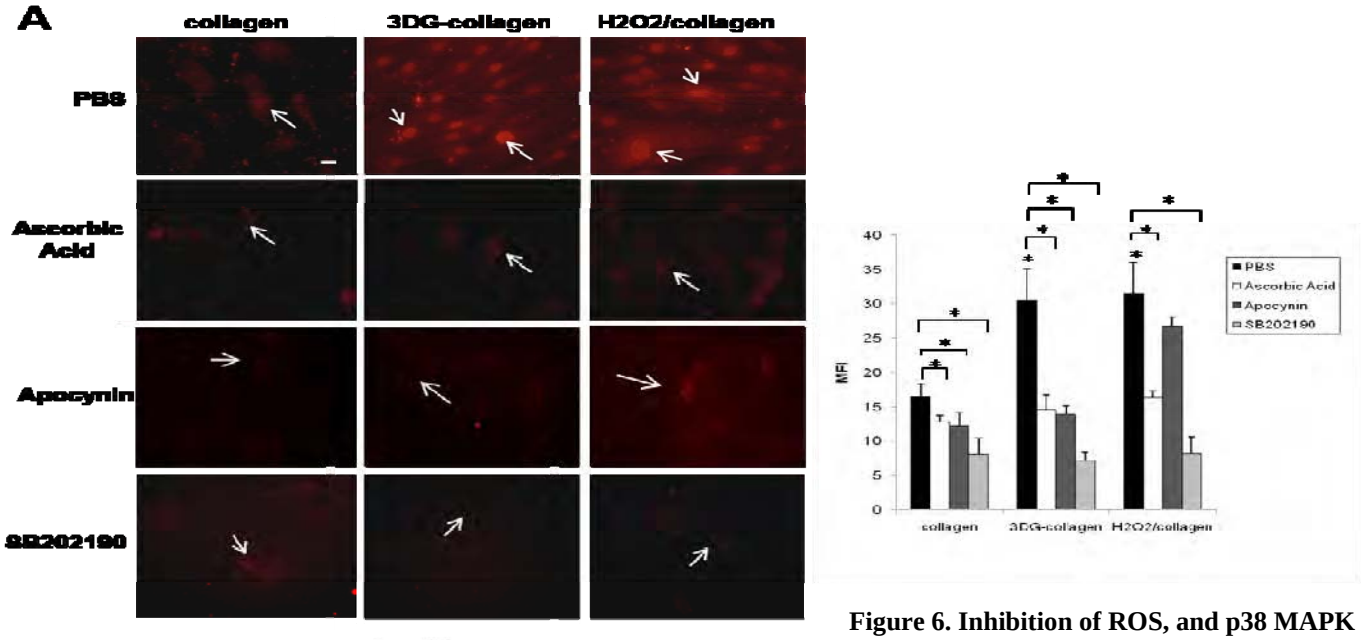


Figure 6. Inhibition of ROS, and p38 MAPK abrogates GADD153 expression in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with 100 µg/mL of ascorbic acid, 1mM apocynin, or 10 µM SB202190 for 1 h, and cultured on native collagen, 1 mM 3DG-collagen or treated with 50 µM H_2O_2 for 24 h. **A**, Fibroblasts were stained and analyzed for the expression of GADD153 in the nucleus by immunofluorescence using a Cy3-conjugated secondary antibody. Representative images were taken at 40 X magnification on an epi-fluorescence microscope, and the MFI of ten nuclei was analyzed by Image J. Bars correspond to the MFI of treated fibroblasts. Arrows indicate nuclei containing GADD153. Scale bar represents 10µm. **B**, Expression of GADD153 protein in whole cell lysates. β-actin was used as a loading control. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean $\pm$ SD (n=3), ***P<0.0001, **P < 0.0005, *P < 0.007.

7. 3DG-collagen-induced caspase-3 activation is dependent on upstream ROS and p38 MAPK activation

A caspase-3 assay was performed to determine if ROS and p38 MAPK are responsible for the increased caspase-3 activation observed in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with ascorbic acid, the Nox inhibitor apocynin, or the p38 MAPK inhibitor SB202190; and cultured on native collagen or 3DG-collagen for 24 h, or cultured on native collagen and treated with H_2O_2 as a positive control. Fibroblasts treated with ascorbic acid, apocynin, or SB202190 and cultured on 3DG-collagen reduced the activation of caspase-3 to 116% $\pm$ 4.7%, 115% $\pm$ 4.5%, and 105% $\pm$ 2.5% respectively. This expression was comparable to the level of caspase-3 cleavage observed in fibroblasts cultured on native collagen and treated with H_2O_2 in the presence of ascorbic acid (112% $\pm$ 7.2%) or SB202190 (104.2% $\pm$ 6.6%), and fibroblasts cultured on native collagen (100% $\pm$ 1.4%; Figure 7; $p < 0.0002$), but not in the presence of apocynin (201% $\pm$ 20.2%). This data suggests that 3DG-collagen is inducing caspase-3 activation through ER stress, which is dependent on upstream activation of ROS and p38 MAPK through upregulation of Nox4. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

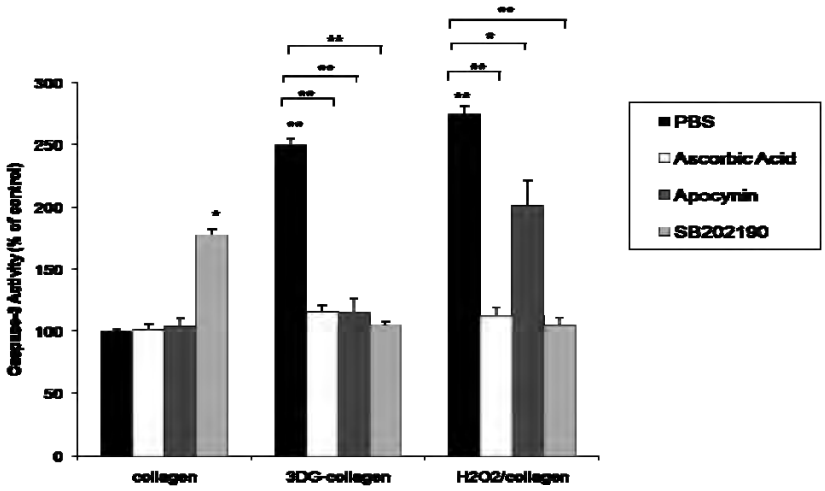


Figure 7. Inhibition of ROS, and p38 MAPK reduces caspase-3 cleavage induced by 3DG-collagen. Fibroblasts were pretreated with 100 µg/mL of ascorbic acid, 1mM apocynin, or 10 µM SB202190 for 1 h and cultured on native collagen, 1 mM 3DG-collagen, or treated with 50 µM H_2O_2 for 24 h. Treatment of fibroblasts with 50 µM H_2O_2 for 24 h was used as a positive control. 100 µg of whole cell lysate was assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean $\pm$ SD (n=3), **P < 0.0002, *P < 0.001.

8. 3DG-collagen induces ROS and apoptosis independent of RAGE signaling.

When AGEs bind to their receptor, RAGE, ROS are released as a downstream byproduct (14). To determine if 3DG-collagen is signaling via its interaction with RAGE, we investigated transcript levels of total RAGE. To our surprise, 3DG-collagen did not upregulate RAGE transcript levels (Figure 8A). As a control for the induction of RAGE we cross-linked collagen with MG, which is a well studied AGE precursor known to signal via RAGE (1;15). We observed a significant induction of RAGE transcript levels in fibroblasts cultured on MG-collagen; 400% $\pm$ 12% upregulation of RAGE (Figure 8A, $p < 0.002$). Additionally, treatment of MG-collagen with AG reduced the transcript levels of RAGE to 119% $\pm$ 13.3%, which confirms that MG can upregulate RAGE mRNA expression. To ensure that 3DG-collagen is not regulating the RAGE receptor post-transcriptionally we measured RAGE protein levels by Western blot. In contrast to the 238% $\pm$ 11.8% upregulation of the RAGE receptor protein in fibroblasts cultured on MG-collagen, 3DG-collagen did not upregulate RAGE protein expression in fibroblasts (107% $\pm$ 10.5%; Figure 8B). To further confirm the specificity of MG, AG abrogated the increase in RAGE protein expression (104% $\pm$ 8.2%) in fibroblasts cultured on MG-collagen (Figure 8B, $p < 0.002$). This data suggests that 3DG-collagen is not upregulating RAGE at both the level of transcription or translation. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

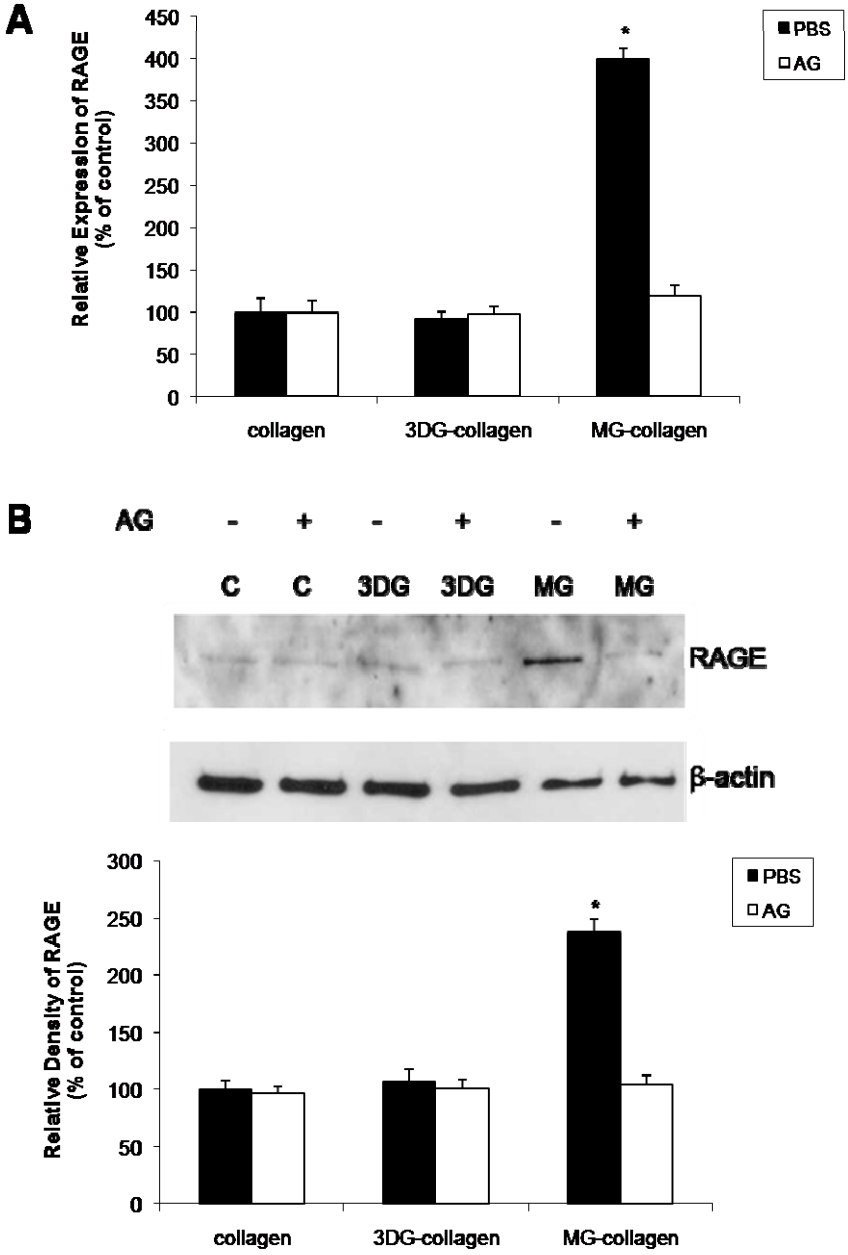


Figure 8. 3DG-collagen does not induce the expression of RAGE. **A**, Fibroblasts were cultured on native collagen, 1 mM 3DG-collagen, or 1 mM MG-collagen with or without 5 mM AG for 24 h. mRNA was analyzed for the expression of RAGE by real-time PCR. All transcripts were normalized to β-actin. **B**, Fibroblasts were treated as in **A** and analyzed for the expression of RAGE by Western blot. The bars correspond to the densitometric value of RAGE after normalization for β-actin. All comparisons are made against collagen treated with PBS. Data are mean ± SD (n=3), *P < 0.002.

To further verify the absence of RAGE expression in 3DG-collagen signaling, RAGE was blocked using a blocking antibody specific for the extracellular domain of RAGE, and the levels of ROS were quantified. Intriguingly, the levels of ROS in fibroblasts cultured on 3DG-collagen did not alter after blockade of RAGE. However, we observed the down regulation of ROS with the inhibition of RAGE binding in fibroblasts cultured on MG-collagen (Figure 9A, $p<0.001$). To further demonstrate that 3DG-collagen signaling was independent of RAGE, we investigated the expression of GADD153. Fibroblasts were pretreated with the RAGE blocking antibody and cultured on native collagen, 3DG-collagen, or MG-collagen; and the level of activated GADD153 was quantified. Blockade of RAGE in fibroblasts cultured on 3DG-collagen did not suppress the activation of GADD153, while GADD153 was suppressed after blockade of RAGE in fibroblasts cultured on MG-collagen, (Figure 9B-C, $p<0.001$). Blockade of RAGE did not decrease the level of caspase-3 activity in fibroblasts cultured on 3DG-collagen, while suppression of

caspase-3 activity was observed in fibroblasts pretreated with the RAGE antibody and cultured on MG-collagen (Figure 9D, $p<0.001$). These results suggest that 3DG-collagen is not signaling through the RAGE receptor as is observed with MG. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

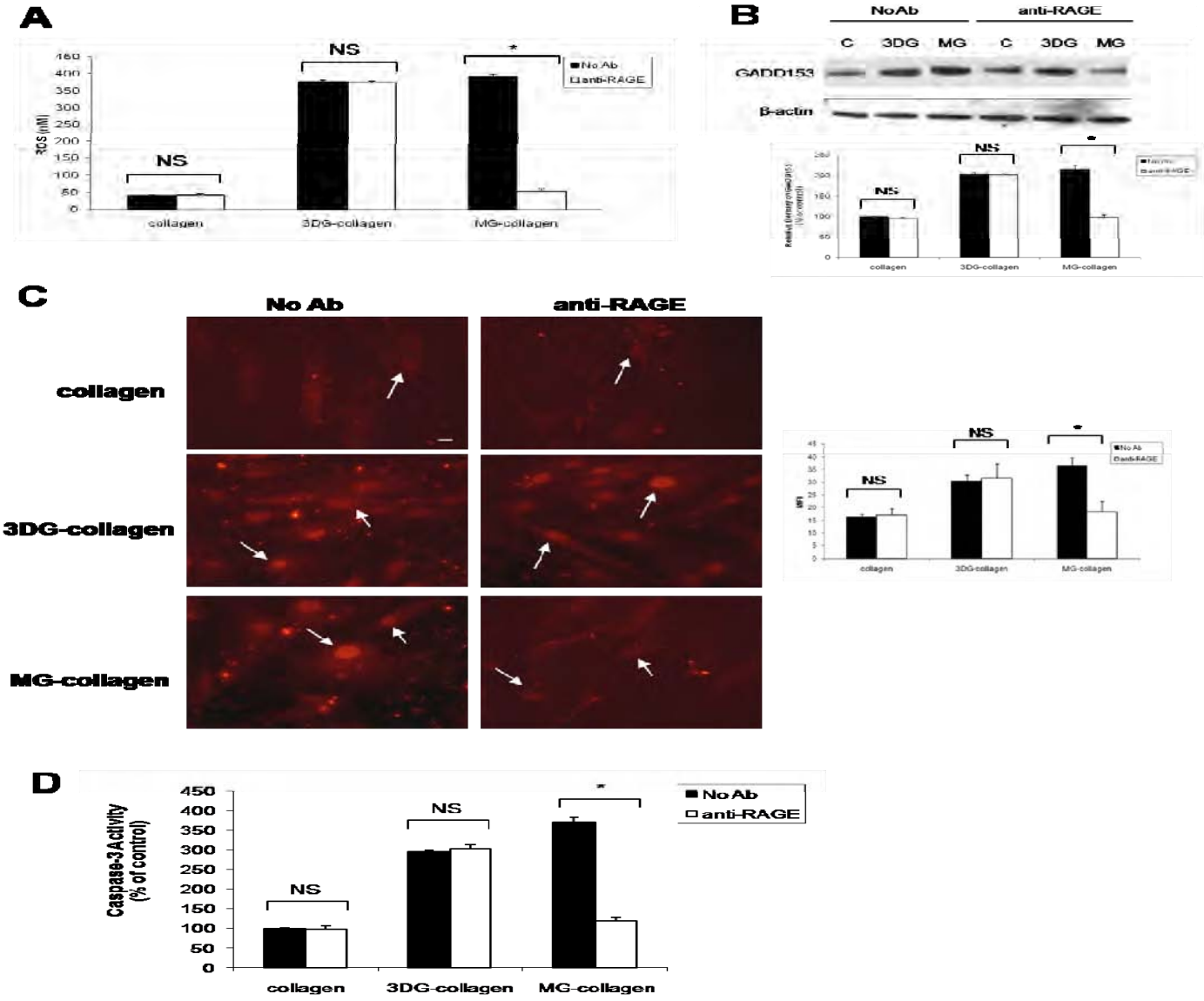


Figure 9. Inhibition of RAGE does not alter the induction of ER stress pathway in fibroblasts cultured on 3DG-collagen. A, Fibroblasts were pretreated with or without the blocking antibody anti-RAGE (10 µg/µL) for 1 h and cultured on native collagen, 1 mM 3DG-collagen, or 1 mM MG-collagen for 24 h and analyzed for the production of ROS. Fibroblasts were incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. **B**, GADD153 protein expression after inhibition of RAGE. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. **C**, GADD153 localization in the nucleus was analyzed by immunofluorescence using a Cy3-conjugated secondary antibody. Images were taken at 40X magnification on an epi-fluorescence microscope and the MFI of ten nuclei was processed by ImageJ. Arrows indicate nuclei containing GADD153. Scale bar represents 10µm. **D**, Caspase-3 activity analyzed according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. Data are mean ± SD (n=3), *P < 0.001.

9. 3DG-collagen activates the ER stress signaling cascade through α1β1 integrin.

To delineate the receptor involved in activating the ER stress pathway by 3DG-collagen, we investigated α1β1 integrin collagen receptor. Previous data has demonstrated that fibroblasts have an increased adherence to 3DG-collagen, which is dependent on binding by α1β1 integrin (6). Fibroblasts can change their binding affinity for 3DG-collagen, which may cause an overproduction of ROS resulting in increased caspase-3 activation. To verify the role of α1β1 integrin on the ER stress signaling pathway, fibroblasts were pretreated with blocking antibodies against either β1 or α1 integrin and the level of ROS was quantified. α5 integrin, the alpha subunit responsible for binding fibronectin, was used as a negative control. Neutralization of both β1 and α1 integrin reduced the production of ROS in fibroblasts cultured on 3DG-collagen to 41.8 nM and 43.2 nM respectively, while neutralization of α5 integrin did not affect production of ROS (Figure 10A, $p<0.001$). We next investigated the effect of β1 and α1 integrin neutralization on the expression of GADD153 in fibroblasts cultured on native collagen or 3DG-collagen for 24h. Blockade of both β1 and α1 integrins suppressed the activation of GADD153 as seen by the decreased expression and nuclear localization (Figure 10B-C, $p<0.001$). Moreover, neutralization of β1 and α1 integrin in fibroblasts cultured on 3DG-collagen reduced the activity of caspase-3 to that observed in fibroblasts cultured on native collagen (Figure 10D, $p<0.001$). These results suggest that the increased binding affinity of α1β1 integrin to 3DG-collagen causes the overproduction of ROS which in turn leads to increased GADD153 activation and cleavage of caspase-3. This data was published in PLOS One in 2010, and a copy of the article has been included in the appendix (5).

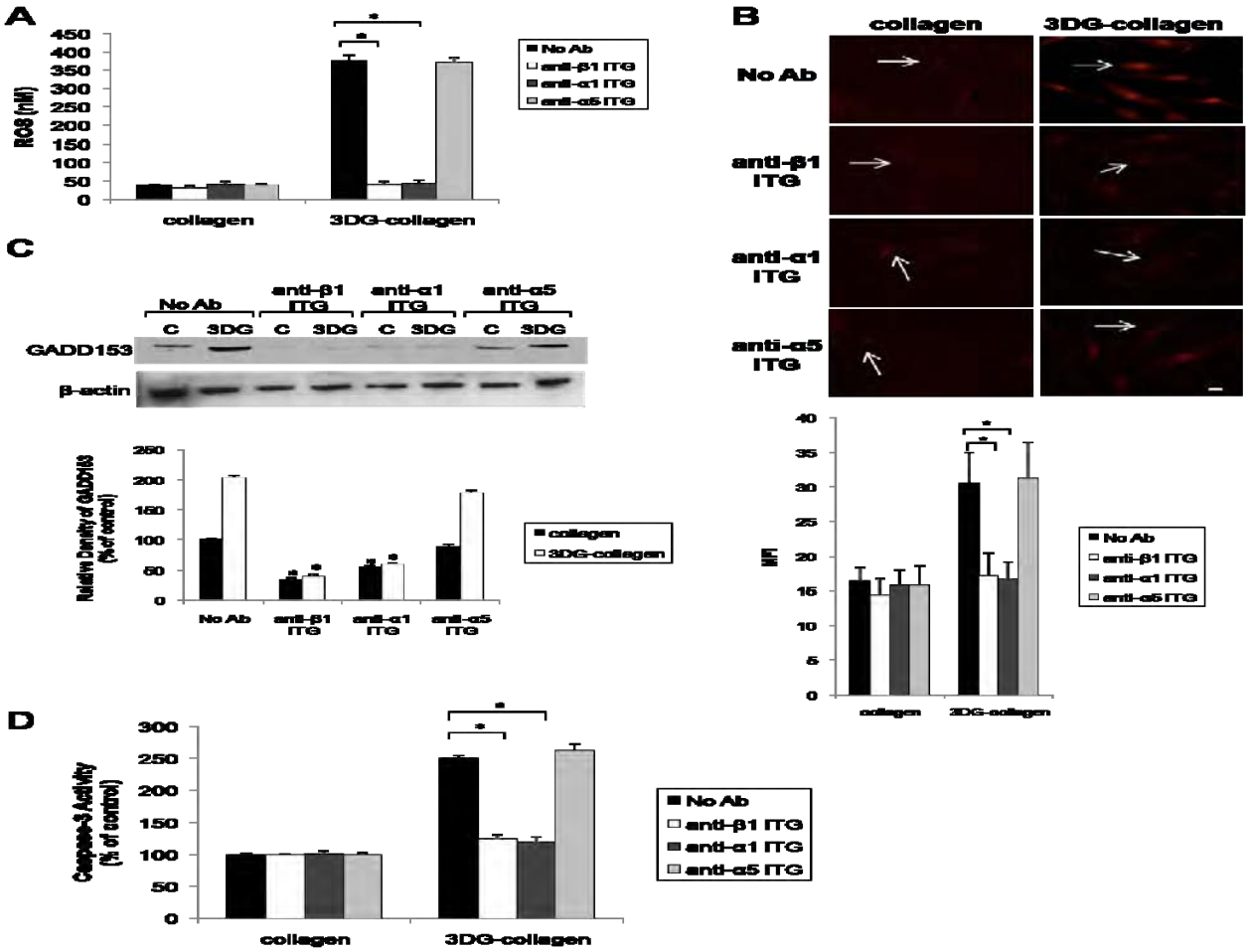


Figure 10. Effect of neutralization of α1β1 integrin on 3DG-collagen-induced ER stress pathway. A, Fibroblasts were pretreated with or without the blocking antibodies anti-β1 ITG, anti-α1 ITG, and anti-α5 ITG (10 μg/μL) for 30 min and cultured on native collagen, or 1 mM 3DG-collagen, for 24 h and analyzed for the production of ROS. Fibroblasts were incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. **B**, GADD153 localization in the nucleus was analyzed by immunofluorescence using a Cy3-conjugated secondary antibody. Images were taken at 40X magnification on an epi-fluorescence microscope and the MFI of ten nuclei was processed by ImageJ. Arrows indicate nuclei containing GADD153. Scale bar represents 10μm. **C**, GADD153 protein expression after neutralization of β1, α1, and α5 integrins. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. **D**, Caspase-3 activity detected using the Caspase-3 Colorimetric Correlate Assay. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), *P < 0.001.

10. HRAS expression inversely correlates with ASK1 expression.

p38 MAPK has been shown to be a key signaling molecule for both HRAS as well as ASK1 (16-19). Evidence has suggested that HRAS activates p38 MAPK to induce cell motility and proliferation (20;21); while, ASK1 activates p38 MAPK under times of stress to induce apoptosis (19;22;23). Because 3DG-collagen is known to induce apoptosis through activation of p38 MAPK (5), we hypothesized that 3DG-collagen could be activating ASK1 while native collagen could promote the phosphorylation of p38 MAPK through the activation of HRAS. To test this, we cultured normal human dermal fibroblasts on native collagen or 3DG-collagen for 24 h, and performed Western blot analysis on the expression of HRAS and phospho-ASK1. Compared to fibroblasts cultured on native collagen, fibroblasts cultured on 3DG-collagen showed a 58% ± 12.4% decrease in the expression of HRAS, with a corresponding 90% ± 10.4% increase in the level of phospho-ASK1 (Figure 11, $p < 0.002$). Additionally, we observed an increase in the level of phospho-p38 MAPK when cells were grown on 3DG-collagen ($p < 0.04$). These data suggest that fibroblasts cultured on native collagen most likely signal through the growth kinase HRAS, while fibroblasts cultured on 3DG-collagen signal through the stress kinase ASK1.

Growth factors are responsible for activating HRAS signaling. Therefore, to determine if blockade of growth factor signaling could alter the expression of HRAS and ASK1 in fibroblasts cultured on native collagen and 3DG-collagen, we employed the use of suramin, a growth factor receptor inhibitor. Fibroblasts pretreated with suramin and cultured on native collagen resulted in a 70%± 10% decrease in the expression of HRAS and an 88% ± 10.8% increase in the phosphorylation of ASK1 (Figure 11, $p < 0.002$). We observed no significant difference in the expression of HRAS or ASK1 between fibroblasts pretreated with suramin and then cultured on 3DG-collagen and fibroblasts cultured on 3DG-collagen alone. These data suggest that the inactivation of growth factor receptors in fibroblasts cultured on native collagen causes HRAS expression to be depressed resulting in the upregulation of ASK1 phosphorylation. Additionally, this response was similar to that observed in fibroblasts cultured on 3DG-collagen. This data is currently under manuscript review.

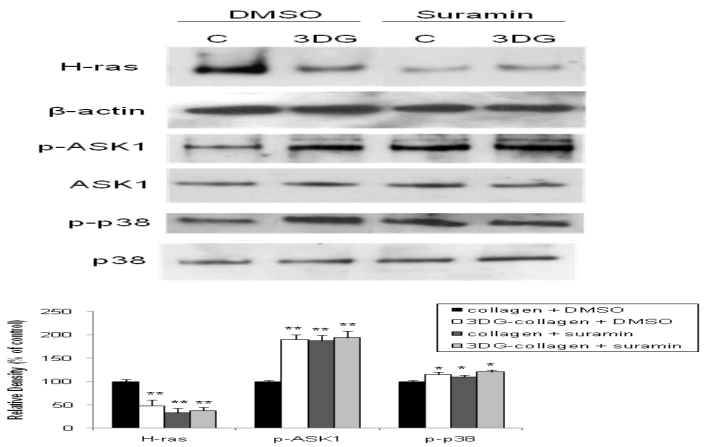


Figure 11. 3DG-collagen and suramin differentially regulate HRAS and phospho-ASK1 expression. Dermal fibroblasts that were 70% confluent were pretreated with suramin or vehicle DMSO for 1 h and cultured on native collagen or 3DG-collagen for 24 h. Using whole cell lysates, expression levels of phospho-p38 MAPK, phospho-ASK1 and HRAS were analyzed by Western blot. Total p38 MAPK, total ASK1, and β-actin served as loading controls. Results were quantified by densitometric scanning of the Western blot and phospho-p38 MAPK was normalized to total p38 MAPK, phospho-ASK1 was normalized to total ASK1, and HRAS was normalized to β-actin. All statistical comparisons are made against native collagen treated with DMSO. Data are mean ± SD (n=3), **P < 0.002, *P < 0.04.

11. p38 MAPK differentially regulates the phosphorylation of ERK1/2 and AKT.

Previous studies have shown that p38 MAPK can cross-talk with other pro-survival kinases, including ERK1/2 and AKT (24). During cell proliferation and migration, p38 MAPK, ERK1/2, and AKT are simultaneously upregulated (25). However, during cellular stress, p38 MAPK can inhibit the phosphorylation of ERK1/2 and AKT resulting in decreased proliferation and migration of the cell and increased apoptosis (24). We have previously shown that 3DG-collagen can downregulate the phosphorylation of ERK1/2 (5); therefore, we investigated if 3DG-collagen-induced p38 MAPK signaling modulated the phosphorylation of ERK1/2 and AKT. Fibroblasts were pretreated with SB202190 (p38 MAPK inhibitor) or the vehicle DMSO for 1 h and then cultured on native collagen or 3DG-collagen for 24 h. Phosphorylation of ERK1/2 and AKT was detected by Western blotting. Inhibition of p38 MAPK with SB202190 in fibroblasts cultured on 3DG-collagen increased the expression of phospho-ERK1/2 from 38% $\pm$ 7.8% to that observed in fibroblasts cultured on native collagen 95% $\pm$ 6.2% (Figure 12, $p < 0.0001$) . Likewise we observed that phospho-AKT increased from 48% $\pm$ 4.4% in cells cultured on 3DG-collagen to 91% $\pm$ 2.4% in fibroblasts cultured on 3DG-collagen with the inhibitor SB202190 (Figure 2, $p < 0.0001$). However, in fibroblasts cultured on native collagen, we observed that inhibition of p38 MAPK down regulated phospho-ERK1/2 to 43% $\pm$ 8.9% and phospho-AKT to 35% $\pm$ 4.8% (Figure 12, $p < 0.0001$) suggesting 3DG changes the crosstalk between p38 MAPK, ERK1/2, and AKT. Pretreatment with suramin reduced the phospho-ERK1/2 and phospho-AKT expression to 37% $\pm$ 6.6% and 46% $\pm$ 8.8%, respectively; in fibroblasts cultured on native collagen (Figure 12, $p < 0.0001$). Moreover, we found that this down regulation of phospho-ERK1/2 and phospho-AKT was dependent on the activation of p38 MAPK as inhibition of this kinase restored the phosphorylation of ERK1/2 and AKT to that observed in control cells (Figure 12). These results suggest that p38 MAPK in the presence of native collagen can act as a growth kinase promoting the phosphorylation of both ERK1/2 and AKT. However, the signaling from p38 MAPK in fibroblasts cultured on 3DG-collagen or in fibroblasts treated with the growth factor receptor inhibitor suramin, altered p38 MAPK to act as a stress kinase resulting in the depression of ERK1/2 and AKT phosphorylation. This data is currently under manuscript review.

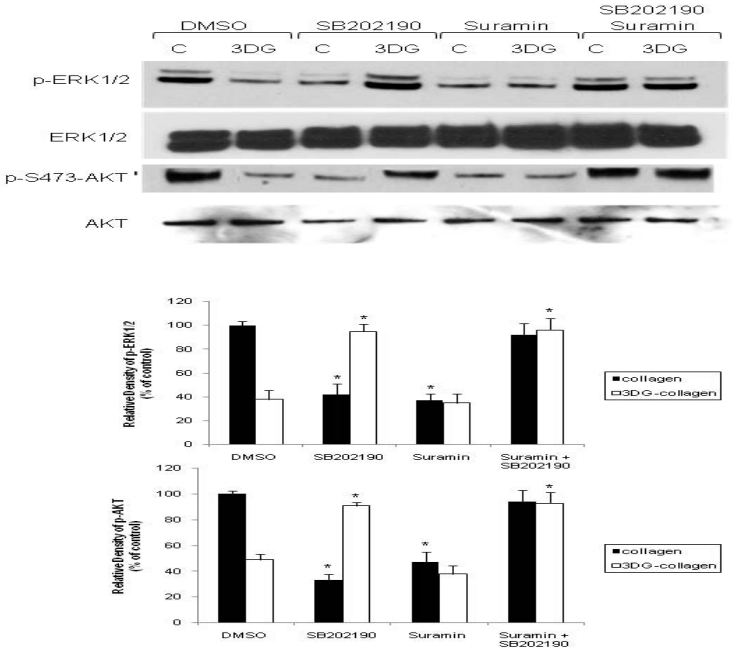


Figure 12. Expression of ERK1/2 and AKT after pretreatment with suramin and p38 inhibitor SB202190. 70% confluent fibroblasts were pretreated with suramin, SB202190, or vehicle DMSO for 1 h and cultured on native collagen or 3DG-collagen for 24 h. Expression of phopsho-ERK1/2 and phospho-AKT were analyzed by Western blot using whole cell lysates. Total ERK1/2 and total AKT served as loading controls. The bars correspond to the densitometric values of phospho-ERK1/2 and phospho-AKT after normalization for total ERK1/2 and total AKT, respectively. All comparisons are made against their respective controls corresponding to native collagen treated with DMSO or 3DG-collagen treated with DMSO. Data are mean $\pm$ SD (n=3), *$P < 0.0001$.

12. **p38 MAPK inversely regulates the migration of fibroblasts cultured on 3DG-collagen or native collagen or when treated with suramin.**

It was previously demonstrated that 3DG-collagen down regulated the migration of dermal fibroblasts in an *in vitro* wound site (5). Since we observed that the inhibition of p38 MAPK in fibroblasts cultured on 3DG-collagen restored the level of phospho-ERK1/2 and phospho-AKT to levels observed in fibroblasts cultured on native collagen, and the phosphorylation of these proteins are known to promote the growth and migration of fibroblasts; we sought to evaluate the significance of p38 MAPK on fibroblast migration. Utilizing an *in vitro* scratch assay, fibroblasts were pretreated with DMSO, suramin, SB202190 (p38 MAPK inhibitor), PD98059 (ERK1/2 inhibitor), or LY294002 (AKT inhibitor) for 1 h and then cultured on native collagen or 3DG-collagen until confluent. A scratch was made along the monolayer of cells and cells were cultured with the inhibitors for an additional 24 h or 48 h. Fibroblasts cultured on native collagen in the absence of any inhibitor had closed the wound by 95% $\pm$ 1.4% by 48 h, while fibroblasts cultured on 3DG-collagen had closed the wound by 68% $\pm$ 2.6% (Figure 13A, $p < 0.0001$). In the presence of the p38 MAPK inhibitor SB202190, fibroblasts cultured on native collagen were unable to efficiently migrate into the wound, resulting in only 67%$\pm$ 3.3% closure by 48 h (Figure 13A, $p < 0.0001$). However, inhibition of p38 MAPK in fibroblasts cultured on 3DG-collagen restored the migration of fibroblasts, closing the wound by 92% $\pm$ 2.2% in 48 h (Figure 13A, $p < 0.001$). Pretreatment of the fibroblasts with the growth factor inhibitor suramin reduced the migration of the fibroblasts cultured on both native collagen and 3DG-collagen resulting in a 64% $\pm$ 3.6% and 61% $\pm$ 2.4% closure of the wound by 48 h, respectively (Figure 13A, $p < 0.0001$). In addition, inhibition of p38 MAPK restored the migration of fibroblasts pretreated with suramin to that observed in the control. These results suggest that p38 MAPK may be acting as a stress kinase in the presence of 3DG-collagen and/or suramin resulting in decreased migration, while p38 MAPK may act as a growth response kinase in fibroblasts cultured on native collagen allowing for fibroblast migration and closure of the wound (Figure 13A, $p < 0.001$). Moreover, the down regulation of HRAS by suramin and 3DG-collagen that we observed in Figure 11 may be contributing to the altered signaling properties of p38 MAPK resulting in decreased cell migration. This data is currently under manuscript review.

To further investigate how p38 MAPK may be altering cellular migration we investigated the growth kinases ERK1/2 and AKT, which are known to regulate wound closure. Fibroblasts pretreated with the ERK1/2 inhibitor PD98059 and cultured on either native collagen or 3DG-collagen did not alter the rate of wound closure compared to that seen in their respective control groups (Figure 13B). Moreover, inhibition of both ERK1/2 and p38 MAPK resulted in wound closure rates similar to that seen in fibroblasts pretreated with the p38 MAPK inhibitor alone. These results suggest that regulation of wound closure by p38 MAPK is not dependent on ERK1/2 activation. Pretreatment of fibroblasts cultured on native collagen with the AKT inhibitor LY294002, closed the wound by only 64% $\pm$ 2.8% in 48 h ($p < 0.001$), and the addition of LY294002 to fibroblasts cultured on 3DG-collagen resulted in a significant reduction in cell migration, as only 6.7% $\pm$ 2.1% of the wound was closed in 48 h (Figure 13C, $p < 0.0001$). The addition of the p38 MAPK inhibitor SB202190 did not further alter the wound closure rates of fibroblasts cultured on native collagen (66% $\pm$ 4.8%) or 3DG-collagen (30% $\pm$ 2.2%) suggesting that p38 MAPK regulation of wound closure is dependent upon the activation of AKT (Figure 13C). This data is currently under manuscript review.

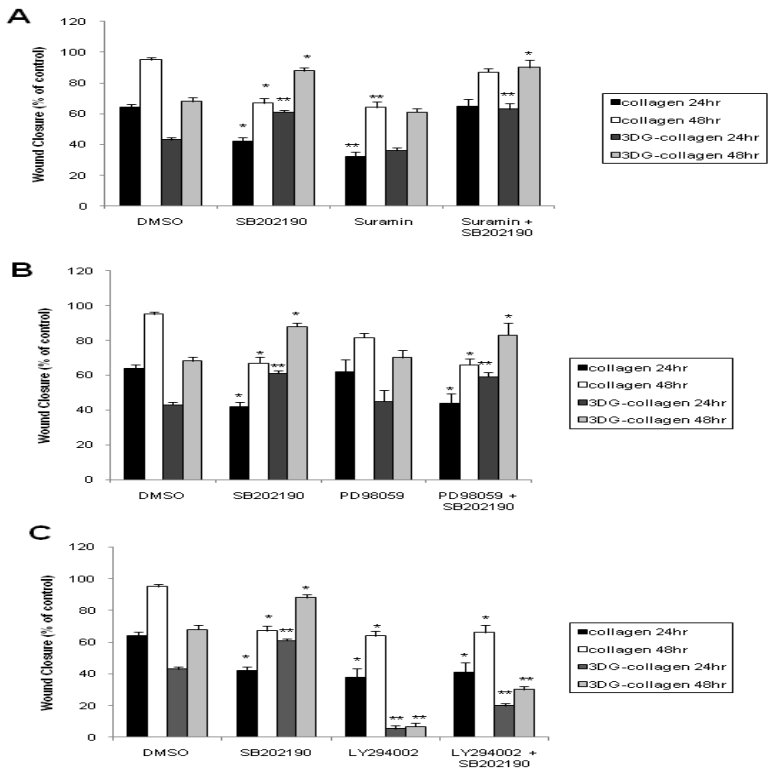


Figure 13. Wound closure rates in fibroblasts pretreated with suramin and kinase inhibitors. Confluent fibroblasts were pretreated with the inhibitors; growth factor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or the ERK1/2 inhibitor PD98059 for 1 h and cultured on native collagen or 3DG-collagen. Cell migration into the wound was monitored at 0 h, 24 h, and 48 h by bright field visualization on an epi-fluorescence microscope. The distance across the wound margin was measured at 10 different points using Spot software and plotted as percentage of wound closure when compared to initial scratch at 0 h. **A**, Wound closure rates of fibroblasts pretreated with suramin and SB202190 and cultured on native collagen and 3DG-collagen. **B**, Wound closure rates of fibroblasts pretreated with SB202190 and PD98059 when cultured on native collagen or 3DG-collagen. **C**, Wound closure rates of fibroblasts pretreated with SB202190 and LY294002 when cultured on native collagen or 3DG-collagen. Statistical comparisons were performed within each time point and compared to their respective controls (native collagen or 3DG-collagen). Data are mean $\pm$ SD (n=3), **P< 0.0001, *P < 0.001.

One of the main migratory features of dermal fibroblasts is the extension of their filopodia along the collagen matrix (26). After mechanical wounding, fibroblasts begin to extend their filopodia into the wound site by 4 h (5). Therefore, we investigated the effect of p38 MAPK on filopodia extension of fibroblasts cultured on native collagen and 3DG-collagen after mechanical wounding. Fibroblasts were pretreated with either the p38 MAPK inhibitor SB202190 or the growth factor receptor inhibitor suramin and the cultured on native collagen or 3DG-collagen until confluent. After confluency, a scratch was made and the actin filaments were stained using rhodamine phalloidin at 4 h post-scratch. As seen previously, fibroblasts cultured on native collagen increased their filopodia by 4 h. In contrast, fibroblasts grown on 3DG-collagen showed minimal extension of their filopodia at 4 h (Figure 14). Inhibition of p38 MAPK with SB202190 delayed filopodia extension into the wound site when fibroblasts were cultured on native collagen. However, inhibition of p38 MAPK induced filopodia extension in fibroblasts cultured on 3DG-collagen (Figure 14). Additionally, suramin reduced the filopodia extension of fibroblasts cultured on native collagen to that seen in 3DG-collagen. Inhibition of p38 MAPK restored the filopodia extension of fibroblasts pretreated with suramin and then cultured on native collagen or 3DG-collagen to that seen in the control (Figure 14). These results suggest that when fibroblasts are cultured on native collagen HRAS dependent activation of p38 MAPK is required for proper filopodia extension, while decreased HRAS may result in ASK1 upregulation of p38 MAPK in fibroblasts grown on 3DG-collagen leading to inhibition of filopodia extension. These results corroborate the current findings that p38 MAPK differentially regulates cellular migration, which is dependent upon the dermal fibroblast's interaction with extracellular stimuli. Moreover, these results are consistent with the findings that p38 MAPK reduces cell migration through down regulation of the phosphorylation of AKT when fibroblasts are cultured on 3DG-collagen as this kinase is needed for proper migration and cell survival. This data is currently under manuscript review.

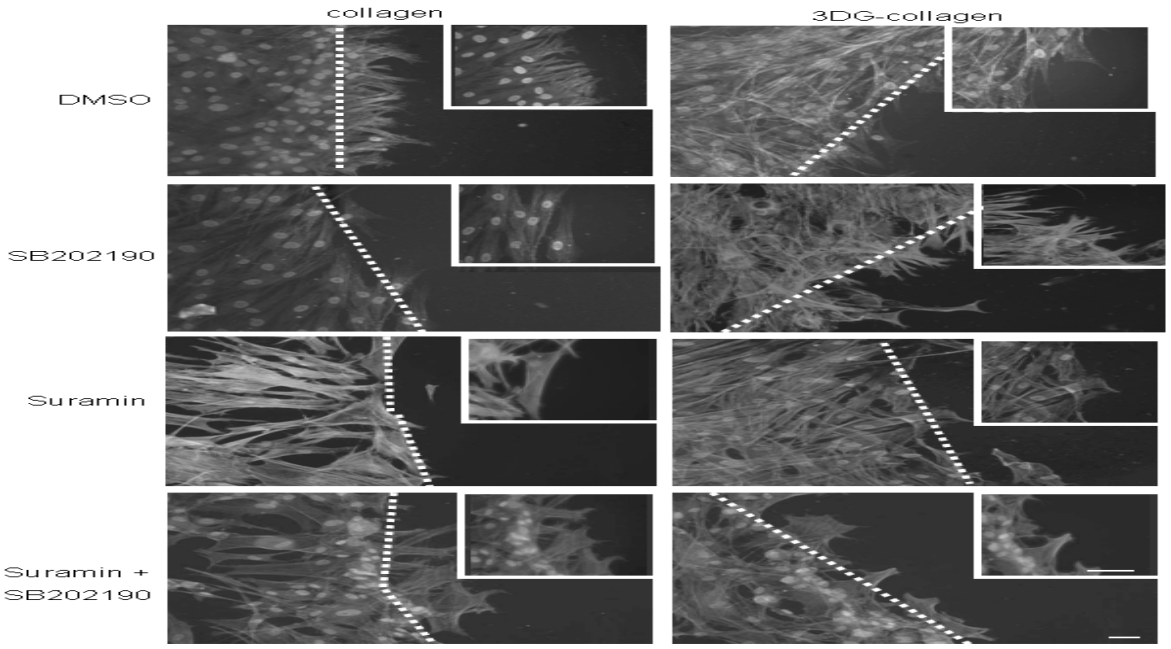


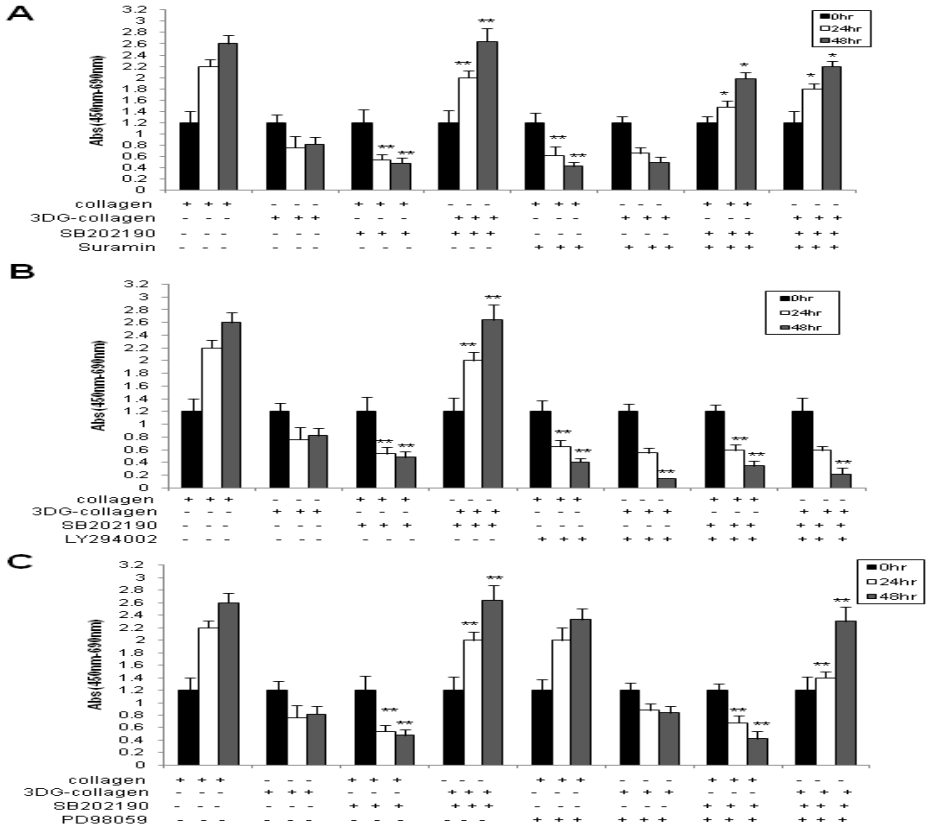
Figure 14. Differential regulation of filopodia extension after fibroblast pretreatment with suramin and p38 MAPK inhibitor SB202190. Fibroblasts were pretreated for 1 h with the inhibitors; growth factor receptor inhibitor suramin, the p38 MAPK inhibitor SB202190, or the vehicle DMSO and then cultured on native collagen or 3DG-collagen until confluent. The monolayer of cells was then manually scratched with a pipette tip to introduce the wound. At 4 h post-scratch the fibroblasts were fixed, permeabilized with Triton X-100, and stained with the F-actin dye rhodamine phalloidin. Extension was denoted as filopodia protrusion from initial wound site. The dotted line denotes initial wound site taken at 20 X magnification. Inset picture taken at 40 X magnification. Scale bar represents 10 μm. 10 images of each sample were taken at 20 X and 40 X magnification, on an epi-fluorescence microscope with an exposure time of 2.2 s. Images represent 1 of 3 independent experiments.

13. Fibroblast proliferation is dependent upon p38 MAPK activation of AKT.

Previous data from our laboratory has shown that 3DG-collagen significantly reduces fibroblast proliferation by 24 h (27). Both ERK1/2 and AKT are known kinases integrally involved in cell proliferation (24). Because p38 MAPK can inversely regulate the expression of these kinases in both a stress and growth environment, we investigated the role of p38 MAPK in cell proliferation. Fibroblasts were pretreated with suramin, SB202190, LY294002, PD98059, or a combination of the inhibitors and cultured on either native collagen or 3DG-collagen. Cell proliferation was measured at 0 h, 24 h, and 48 h. Fibroblasts cultured on native collagen steadily proliferate over 48 h, while fibroblasts cultured on 3DG-collagen show a decrease in their proliferative capacity within 48 h (Figure 15A, $p < 0.001$). There was a down regulation in the rate of proliferation when p38 MAPK was inhibited in fibroblasts cultured on native collagen compared to control. When p38 MAPK was inhibited with SB202190 in fibroblasts cultured on 3DG-collagen, proliferation was restored. These fibroblasts had proliferated to similar numbers at 48 h to that seen in fibroblasts cultured on native collagen. Pretreatment with suramin reduced the fibroblast's ability to proliferate on native collagen similar to that seen in fibroblasts cultured on 3DG-collagen. Furthermore, with the addition of the p38 MAPK inhibitor SB202190 to fibroblasts pretreated with suramin, the proliferation rate was restored to that observed in fibroblasts cultured on native collagen suggesting that down regulation of HRAS may alter the phenotype of p38 MAPK (Figure 15A, $p < 0.03$).

Next we investigated what kinase p38 MAPK was regulating to induce proliferation. Fibroblasts were pretreated with the ERK1/2 inhibitor PD98059 and the AKT inhibitor LY294002 and proliferation was measured over 48 h. We found that the rate of proliferation in fibroblasts cultured on native collagen was

dependent on AKT but not ERK1/2 as only inhibition of AKT showed similar levels of proliferation to that seen in fibroblasts pretreated with the p38 MAPK inhibitor (Figure 15B, C; $p<0.001$). Moreover, the simultaneous inhibition of p38 MAPK and AKT did not provide any significant additive effect to the decreased rate of proliferation seen in these fibroblasts, suggesting that proliferation is dependent on the p38 MAPK regulation of AKT (Figure 15B, $p<0.001$). In addition, fibroblasts pretreated with AKT alone and cultured on 3DG-collagen showed no signs of proliferation in the first 24 h and by 48 h the majority of the cells had died, while there was no change in the proliferation capacity of these cells when ERK1/2 was inhibited (Figure 5B, C; $p<0.001$). These studies suggest that 3DG-collagen-induced p38 MAPK downregulates cell proliferation by down regulating the phosphorylation of AKT. This data is currently under manuscript review.



14. AKT activation is required for regulation of caspase-3 by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen.

ASK1 is known to activate p38 MAPK to be a pro-apoptotic kinase signaling the activation of apoptotic signaling cascades (19). However, recent studies have shown that HRAS-dependent upregulation of p38 MAPK can enhance cell survival in cancer cells through activation of AKT (20). 3DG-collagen has been shown to induce the expression of caspase-3 (5). Therefore, we investigated the role of p38 MAPK in caspase-3 activation under growth and stress conditions. When fibroblasts were pretreated with the p38 MAPK inhibitor SB202190 and cultured on native collagen, there was a 77% $\pm$ 5.2% increase in the level of caspase-3 activation (Figure 16, $p < 0.001$). Moreover, when fibroblasts were pretreated with suramin, which is known to increase ASK1, and grown on native collagen there was an increase in caspase-3 activation to 285%$\pm$ 10.2% and this was dependent on the activation of p38 MAPK (Figure 16, $p < 0.0002$). To determine how p38 MAPK is regulating caspase-3 activation in fibroblasts cultured on native collagen we investigated the role of AKT and ERK1/2. The regulation of caspase-3 by p38 MAPK in fibroblasts cultured

on native collagen was dependent on the activation of AKT as inhibition of AKT increased the level of caspase-3 to 189% ± 2.4% ($p < 0.001$). Additionally, there was no significant additive effect on the level of caspase-3 activation when both p38 MAPK and AKT were simultaneously inhibited (207% ± 3.2%). Moreover, the inhibition of ERK1/2 did not significantly increase the expression of caspase-3 (109% ± 3.3%), while inhibition of both ERK1/2 and p38 MAPK increased the expression of caspase-3 to 181% ± 4.6% suggesting that ERK1/2 is not responsible for the survival of the cell (Figure 16, $p < 0.001$). Instead, survival of fibroblasts cultured on native collagen is dependent upon p38 MAPK-induced AKT activation. This data is currently under manuscript review.

We next investigated the role of p38 MAPK in fibroblasts cultured on 3DG-collagen. As seen previously, fibroblasts cultured on 3DG-collagen increased the expression of caspase-3 to 250%± 4.5% and this upregulation was abrogated when p38 MAPK was inhibited (Figure 16, 105% ± 2.5%, $p < 0.0002$). Moreover, pretreatment of fibroblasts cultured on 3DG-collagen with suramin upregulated caspase-3 expression to that seen in fibroblasts cultured on 3DG-collagen alone, and this upregulation was dependent on p38 MAPK. These results suggest that suramin may be utilizing p38 MAPK in the same manner as 3DG-collagen to reduce fibroblast cell survival.

As shown previously p38 MAPK downregulates the phosphorylation of AKT and ERK1/2 in fibroblasts cultured on 3DG-collagen (Figure 12); therefore, we investigated whether down regulation of AKT or ERK1/2 by p38 MAPK was responsible for increased caspase-3 activation in fibroblasts cultured on 3DG-collagen. The activation of caspase-3 by p38 MAPK in fibroblasts cultured on 3DG-collagen is shown to be dependent on the inactivation of AKT. Inhibition of AKT caused a 189% ± 6.2% increase in the level of caspase-3 activation in fibroblasts cultured on 3DG-collagen (Figure 16, $p < 0.0002$). This increase was not significantly altered when fibroblasts were pretreated with both AKT and p38 MAPK inhibitors simultaneously (165% ± 5.2%), unlike that seen in fibroblasts treated simultaneously with ERK1/2 and p38 MAPK inhibitors (155% ± 6.5% increase with ERK1/2 inhibitor vs. 10% ± 4.2% increase with ERK1/2 and p38 MAPK inhibitor). These results suggest that in fibroblasts cultured on 3DG-collagen p38 MAPK reduces the phosphorylation of AKT, which is responsible for increased caspase-3 activation. This data is currently under manuscript review.

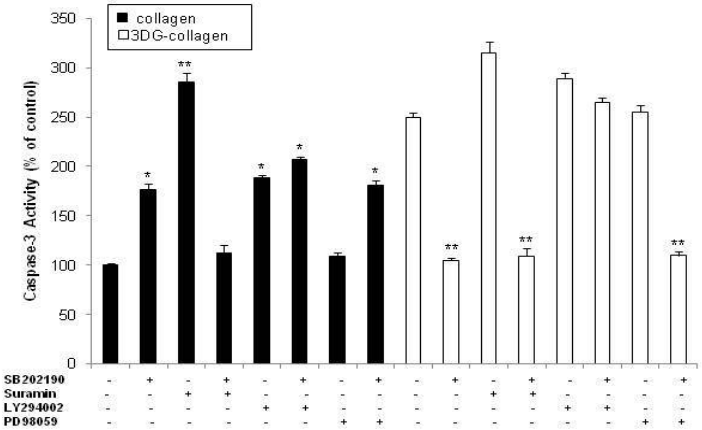


Figure 16. Measurement of caspase-3 activation after fibroblast pretreatment with suramin and kinase inhibitors. Fibroblasts were pretreated with the inhibitors; growth factor receptor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then cultured on native collagen or 3DG-collagen for 24 h. Whole cell lysates were assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against their respective controls (native collagen or 3DG-collagen alone). Data are mean ± SD (n=3), **P < 0.0002, *P < 0.001.

15. Type I collagen expression is inversely regulated by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen.

During wound healing, type I collagen is synthesized by dermal fibroblasts to aid in successful contraction of the wound margins (28). p38 MAPK has been shown to reduce collagen production in fibroblasts explanted from diabetic wounds (29) and we have demonstrated that 3DG-collagen inhibits the

expression of type I collagen by the fibroblast (27); therefore, we investigated the role of p38 MAPK on type I collagen production. Fibroblasts were pretreated for 1 h with the inhibitors suramin, SB202190, LY294002, PD98059, or a combination; and cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts cultured on native collagen induced the expression of collagen at both the level of transcription and translation. In contrast, fibroblasts cultured on 3DG-collagen reduced both the transcript levels of COL1A1 (75%$\pm$ 3.2%) and the protein levels of procollagen (62%$\pm$ 4.2%). Inhibition of p38 MAPK with SB202190 in fibroblasts cultured on native collagen showed both reduced transcript levels of COL1A1 (68%$\pm$ 3.2%, $p < 0.0001$) and reduced expression of procollagen (62%$\pm$ 6.5%, $p < 0.0001$). In contrast, inhibition of p38 MAPK restored the expression of COL1A1 (101%$\pm$ 4.2%) and procollagen (98%$\pm$ 4.4%) in fibroblasts grown on 3DG-collagen (Figure 17A, B; $p < 0.0001$). Inhibition of growth factor receptors by suramin reduced the mRNA levels of COL1A1 to 71%$\pm$ 7.8% and the protein content of procollagen to 68%$\pm$ 7.2% in fibroblasts cultured on native collagen. This effect was similar to that observed in fibroblasts cultured on 3DG-collagen (Figure 17A, B; $p < 0.0001$). Moreover, this effect was found to be dependent on the activation of p38 MAPK as inhibition of p38 MAPK in fibroblasts pretreated with suramin and cultured on native collagen abrogated the down regulation of COL1A1 (95%$\pm$ 9.8%, $p < 0.0001$) and procollagen (90%$\pm$ 10.0%, $p < 0.0001$).

p38 MAPK regulation of collagen is dependent on the activation of both AKT and ERK1/2 as there was a similar decrease in collagen expression when both p38 MAPK and AKT and p38 MAPK and ERK1/2 were inhibited in fibroblasts grown on native collagen (Figure 17A, B; $p<0.0001$). In fibroblasts cultured on 3DG-collagen, collagen expression was dependent on the p38 MAPK down regulation of both AKT and ERK1/2 as restoration of collagen expression by p38 MAPK inhibition was abrogated when both AKT and ERK1/2 were independently inhibited (Figure 17A, B; $p < 0.0001$). These data suggest that p38 MAPK is playing a major role in the transcription and translation of collagen. p38 MAPK is playing a positive role in the regulation of collagen when fibroblasts are grown on native collagen, while it plays a negative role in fibroblasts grown on 3DG-collagen. This data is currently under manuscript review.

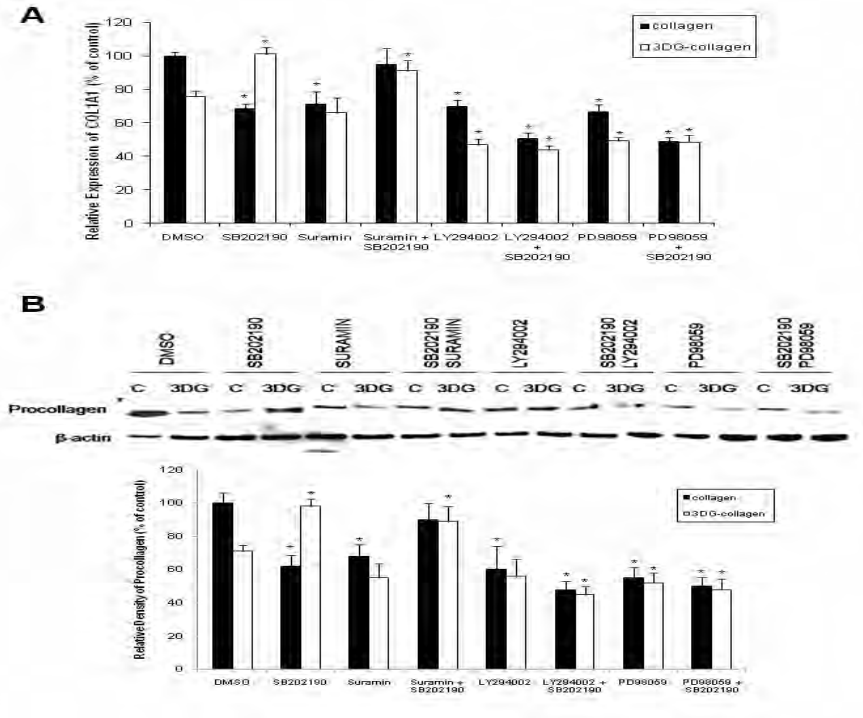


Figure 17. Expression of type I collagen after fibroblast pretreatment with suramin and kinase inhibitors. Fibroblasts were pretreated with the inhibitors; growth factor receptor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then cultured on native collagen or 3DG-collagen for 24 h. **A**, COL1A1 mRNA expression levels were quantified by real-time RT-PCR. All transcripts were normalized to β-actin. **B**, Expression levels of procollagen were analyzed by Western blot and β-actin served as a loading control. The bars correspond to the densitometric value of procollagen after normalization for β-actin. All comparisons are made against their respective controls (native collagen or 3DG-collagen). Data are mean $\pm$ SD (n=3), *$P < 0.0001$.

16. **Can Type I collagen Expression be Decreased by the Signaling Inhibitors, SB202190, LY294002, Ras, Atiprimod, or suramin in SSc Fibroblasts?** Because 3DG-collagen induced a decrease in collagen expression in both normal and SSc fibroblasts and because we know the signaling pathways mediated by 3DG-collagen, we investigated the role of the inhibitors suramin (growth factor receptor), SB202190 (p38 MAPK inhibitor), LY294002 (AKT inhibitor), a Ras inhibitor, and Atiprimod a STAT3 inhibitor and determined collagen expression in SSc. We have made the following observations suggesting that these pathways are also operational in SSc fibroblasts and that inhibitors of these signaling molecules could be used to decrease collagen expression and therefore make the quality of life better in SSc patients. We found that the AKT inhibitor LY294002 worked slightly better than the p38 MAPK inhibitor SB202190.

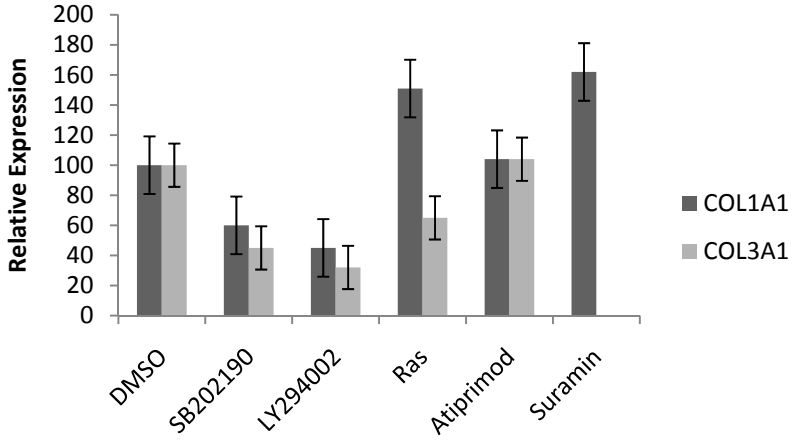


Figure 18. Collagen expression in SSc fibroblasts treated with SB202190, LY294002, Ras, Atiprimod, or Suramin. Confluent SSc fibroblasts were pretreated with the inhibitors; p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, Ras inhibitor, Atiprimod a STAT3 inhibitor, or Suramin a growth factor receptor inhibitor for 24 h. RNA was isolated and COL1A1 and COL3A1 transcripts measured by real-time PCR. DMSO was used as a control for the carrier solution. We found that SB202190 and LY294002 decreased collagen expression, whereas the Ras, Atiprimod, and suramin did not.

We further elucidated the signaling in SSc fibroblasts and determined that it is not operating through the Ras/MEK/ERK pathway. We found that when the Ras inhibitor or suramin was added to the SSc fibroblasts collagen expression was increased. This suggests that the signal is not coming from growth factor receptors but from the TGFβ1 receptor that directs signaling via AKT and p38. We have also investigated the inhibitor Atiprimod. This inhibitor targets STAT3 which receives its signal from JAK. JAK signaling can also affect AKT signaling. Indeed, on analysis of Atiprimod, we found collagen expression did not alter in SSc fibroblasts, further confirming the importance of the AKT pathway and that STAT3 did not contribute to SSc fibroblast signaling (Figure 18).

17. **Flow Cytometry of Integrin Subunit Expression on SSc fibroblasts Cultured on 3DG-collagen.** We have performed competition assays to determine which integrins are more important for binding to 3DG collagen. We found that the expression of the integrins α1, α5, αv, and β1 were increased in whole cell lysates and were also increased on the cell surface by flow cytometry. However we found that β1 integrin was decreased in whole cell lysates but increased on the cell surface. Indeed, SSc fibroblasts have decreased α1β1 integrin which correlates with increased collagen expression(30) and here we found that 3DG-collagen increased α1β1 integrin on the cell surface measured by Flow cytometry thus decreasing the expression of collagen. We suspect that 3DG-collagen induces integrin recycling (31). We are further elucidating this observation.

18. **Aim 3. Modulation of the formation of 3DG by Dyn15 (meglumine), Dyn24 (morpholinofructose), fructoselysine, and isoquercitrin and their subsequent effects on ECM expression.** We have purchased isoquercitrin and have started the final studies proposed in this application. We have well-defined studies that demonstrate the increased expression of collagen and cell proliferation with meglumine. One observation that we find to be intriguing is that fructoselysine added to the fibroblasts

induced a greater decrease in collagen expression compared to the same amount of 3DG added to fibroblasts (Figure 19). We speculate that because fructoselysine is metabolized to 3DG, it reacts directly within the cell. 3DG is taken up by the cells but as it is highly reactive, it may have reacted to itself; therefore a compound that could be metabolized by cells may have more therapeutic benefit.

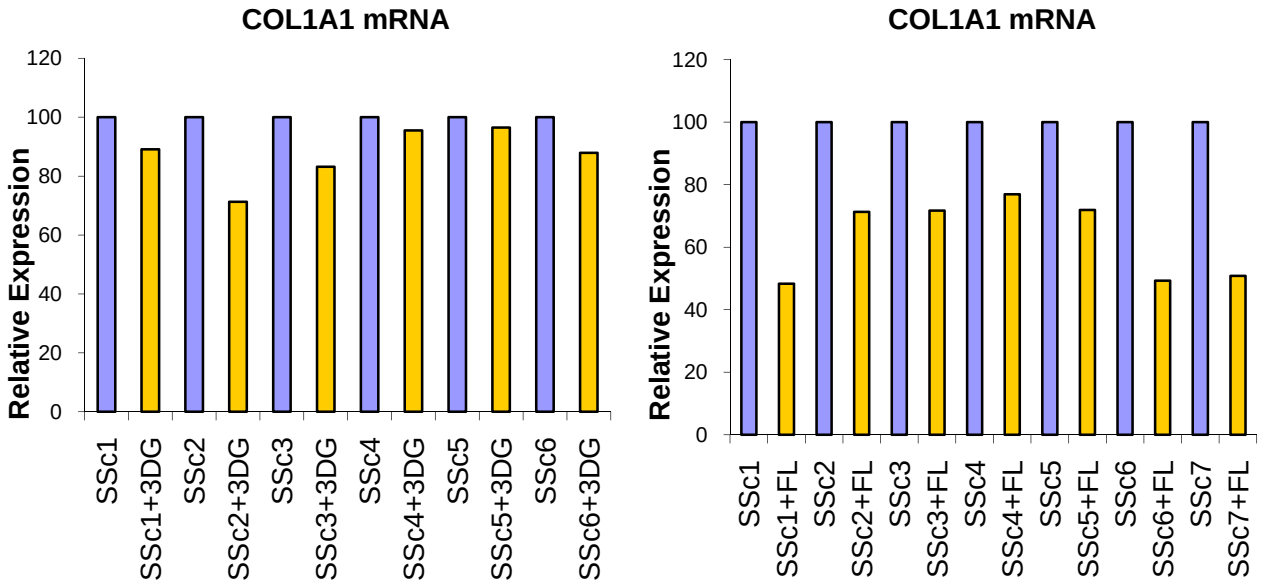


Figure 19. Decreased expression of COL1A1 with 3DG and fructoselysine. SSc fibroblasts were cultured with 1 mM 3DG or 1 mM fructoselysine for 24 h. Cells were lysed, cDNA made and COL1A1 transcripts measured by SYBR Green amplification. Mean decrease in COL1A1 was found to be 12.75% with 3DG, whereas it was 37.12% with fructoselysine. These differences were statistically significant $P < 0.0001$.

Additionally, in the last few months we have developed an analog of meglumine, termed Dyn35. Dyn35 is more efficient at inducing collagen expression and wound closure at therapeutic (μM) doses. We are taking this data further to develop Dyn35 as a topical ointment for chronic wounds that could be utilized for diabetic ulcers.

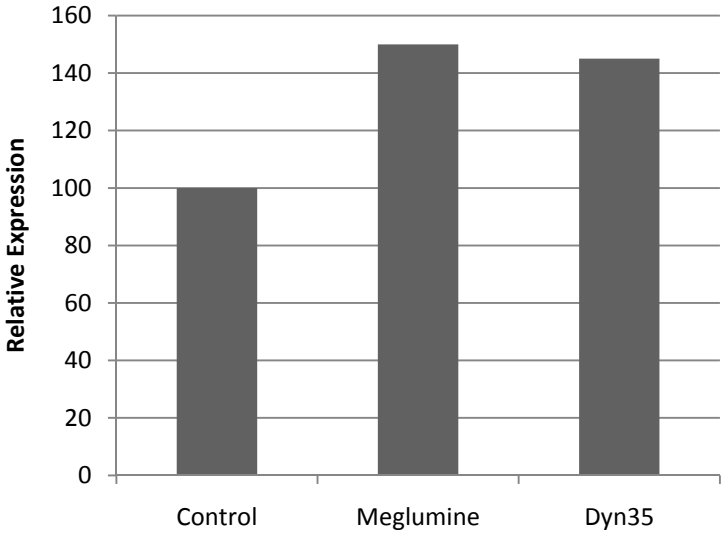


Figure 20. COL1A1 Expression in fibroblasts treated with meglumine and Dyn35. Fibroblasts were treated with 40 mM meglumine and 10 μM Dyn35 for 24 h. Cells were lysed and COL1A1 cDNA transcripts were measured by real-time PCR with SYBR green. We have found that micromolar doses of Dyn35 will produce the same effect as millimolar dose of meglumine.

Furthermore, we are in the process of developing anti-fibrotic treatments for SSc based on the data we have acquired. We know that 3DG cannot be utilized for therapy as it is a toxic byproduct of glucose metabolism, therefore the data we have acquired on the alterations of signaling in fibroblasts due to 3DG-collagen

K**ey Research Accomplishments for June 2009 – May 2010.**

We have found that 3DG affects the following parameters in fibroblasts which results in the decreased expression of collagen.

1. 3DG-collagen induces caspase-3 expression via ER stress (Figure 1)
2. 3DG-collagen induction of ER stress induces GADD153 (Figure 2)
3. 3DG-collagen stimulates reactive oxygen species (Figure 3)
4. NAD(P)H oxidase 4 is responsible for 3DG-collagen dependent production of reactive oxygen species (Figure 4)
5. 3DG-collagen induced phosphorylation of p38 MAPK is dependent on upstream reactive oxygen species (Figure 5)
6. 3DG-collagen induced GADD153 expression is dependent on upstream reactive oxygen species and p38 MAPK (Figure 6)
7. 3DG-collagen induced caspase-3 activation is dependent on upstream reactive oxygen species and p38 MAPK activation (Figure 7)
8. 3DG-collagen induces reactive oxygen species and apoptosis is independent of RAGE signaling (Figure 8 and Figure 9)
9. 3DG-collagen activates the ER stress signaling cascade through α1β1 integrin (Figure 10)
10. HRAS expression inversely correlates with ASK1 expression in fibroblasts cultured on 3DG-collagen (Figure 11)
11. P38 MAPK differentially regulates the phosphorylation of ERK1/2 and AKT (Figure 12)
12. P38 MAPK inversely regulates the migration of fibroblasts cultured on 3DG-collagen or native collagen, or when treated with suramin (Figure 13 and 14)
13. Fibroblast proliferation is dependent on p38 MAPK activation of AKT (Figure 15)
14. ALT activation is required for regulation of caspase-3 by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen (Figure 16)

15. Type I collagen expression is inversely regulated by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen (Figure 17)
16. Type I collagen expression can be decreased in SSc fibroblasts with the p38 MAPK and AKT inhibitors (Figure 18)
17. Fructoselysine is more effective at reducing collagen expression than 3DG (Figure 19.
18. Dyn35, an analog of Dyn15/meglumine can be used at therapeutic doses to increase collagen expression (Figure 20). We are developing this analog to treat diabetic ulcers which do not have enough collagen expression.

Reportable Outcomes

Publications

1. Loughlin DT, Artlett CM. 3-Deoxyglucosone-Collagen Alters Human Dermal Fibroblast Migration and Adhesion: Implications for Impaired Wound Healing in Patients with Diabetes. Wound Repair Regen **17**:739-749, 2009.
2. Sassi-Gaha S, Loughlin DT, Kappler F, Schwartz ML, Su B, Tobia AM, Artlett CM. Two dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts. Matrix Biology **29**:127-134, 2010.
3. Loughlin DT, and Artlett CM. Precursor of Advanced Glycation End Products 3-deoxyglucosone Mediates ER-stress-induced Caspase-3 Activation of Human Dermal Fibroblasts through NAD(P)H Oxidase 4. PLOS One **5**: e11093, 2010.

Abstract Presentation at Meetings

1. Loughlin DT, Sassi-Gaha S, Artlett CM. 3-Deoxyglucosone-modified collagen induces apoptosis of dermal fibroblasts through activation of GADD153 via p38 MAPK. Discovery Day 2009. DT Loughlin won the PhD student competition with her podium presentation.
2. Artlett CM, Loughlin DT, Feghali-Bostwick CA, Sassi-Gaha S. Decreased extracellular matrix molecules in systemic sclerosis fibroblasts utilizing the glucose metabolite 3-deoxyglucosone. Military Health Research Forum, Kansas City 2009.

Conclusion. We have elucidated many signaling steps induced by 3DG-collagen that results in the decreased collagen expression observed by this molecule and started testing possible therapeutic inhibitors for signaling pathways affected. These findings allow us to further elucidate their effect in SSc and also to identify suitable inhibitors that could be developed therapeutically as a treatment for SSc that will result in the decreased expression of collagen. Based on these findings, we have identified several commercially available inhibitors of these pathways that may be more suitable for the treatment of SSc including SB202190 and LY294002. Furthermore, we have identified sources where we can employ compounds that will promote the development of 3DG, whereby decreasing the expression of collagen intracellularly. In addition, these studies have allowed us to identify suitable compounds that will enhance collagen expression and wound closure

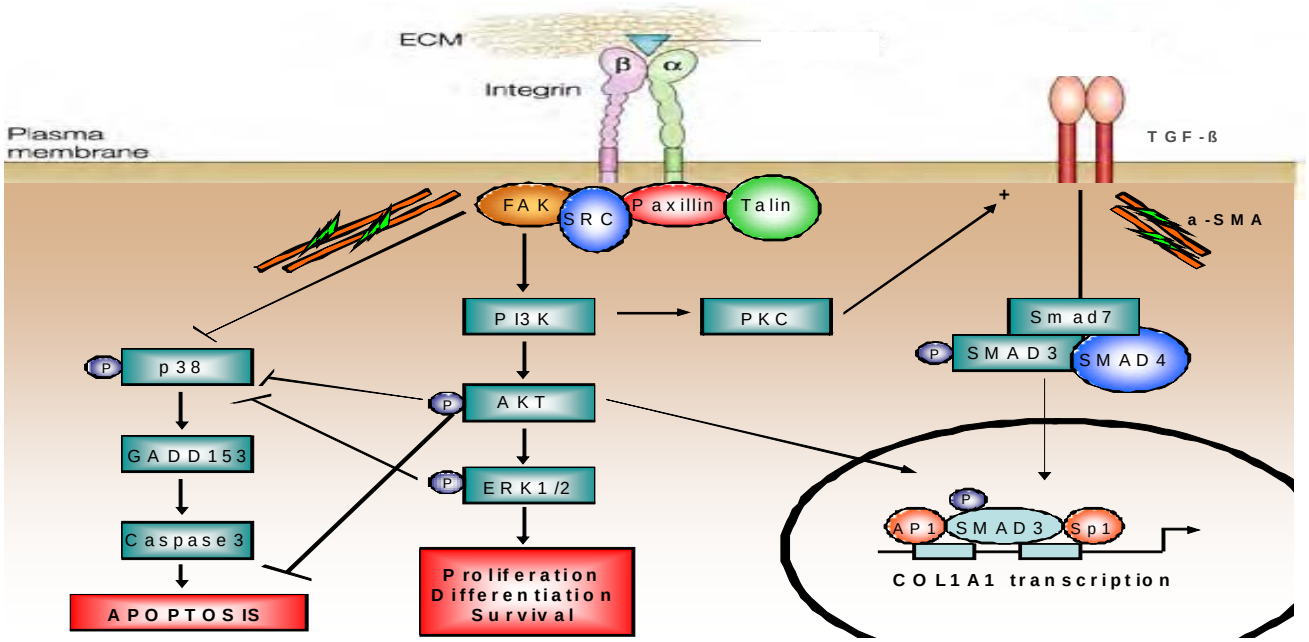


Figure 21. Cell Signaling Altered by 3DG-collagen.

Appendices

Enclosed is a current copy of the IRB approval for this study. Please note that ongoing IRB approval is no longer necessary for this study as it is an exempt research protocol without HIPAA. See enclosed letter.

Published articles have been included.

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Title: 3DG-modified collagen induces apoptosis of dermal fibroblasts through activation of GADD153 via p38 MAPK

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Abstract:

Wound healing is impaired in patients with diabetes as approximately 5-8% of the patients develop chronic foot ulcers. In the context of diabetes, the precursor for advanced glycation end products (AGEs) 3-deoxyglucosone (3DG) is highly elevated and may contribute to the impaired function of fibroblasts. We have shown that 3DG-collagen can induce apoptosis, which may explain the high degree of cell death seen in fibroblasts explanted from the skin of diabetic patients. Previous results have revealed a role for the growth arrest and DNA damage-inducible gene (GADD153) in 3DG-collagen-induced apoptosis. However, the mechanism is unclear, prompting this investigation to further clarify the effects of 3DG-collagen on the induction of apoptosis in human dermal fibroblasts. We found that 3DG-collagen induced apoptosis through induction of GADD153, which was dependent on upstream ROS and p38 activation. 3DG-collagen also downregulated the anti-apoptotic kinase Akt and the growth kinase ERK 1/2, which was partially dependent on the activation of p38. Additionally on 3DG-collagen, inhibition of p38 partially restored fibroblast migration into the wound site, while its inhibition abrogated fibroblast migration on native collagen. An inhibitor of 3DG, meglumine decreased ROS and showed low levels of GADD153 and apoptosis suggesting that meglumine acts by decreasing intracellular free radicals. These results provide a foundation for the signaling elicited between the fibroblast and the 3DG-modified collagen and may yield clues to the poor wound healing capacity observed in patients with diabetes. By elucidating this signaling pathway, new therapeutics may become available for the increasing population suffering from chronic wounds.

Decreased Extracellular Matrix Molecules in Systemic Sclerosis Fibrotic Fibroblasts Utilizing the Glucose Metabolite 3-Deoxyglucosone (3DG)

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[1]Microbiology & Immunology, Drexel University College of Medicine; [2]Lung Pathology, University of Pittsburgh

Background and Objectives: Scleroderma (SSc) is a fibrotic disease of unknown origin that affects morbidity and leads to mortality in these patients. The fibroblast balances ECM expression through interactions with integrin (ITG), mediating excessive collagen production as seen in fibrotic disorders or alternatively too little collagen as seen in chronic wounds. Glucose metabolites crosslink collagen and these crosslinks accumulate on the collagen as a function of age and diet. 3DG has been implicated in diabetic complications such as chronic wounds. As glucose metabolites appear to be associated with chronic wounds, we investigated ECM expression the signaling within the fibroblasts to determine if 3DG modulates collagen expression in SSc fibroblasts.

Methodologies: Primary dermal cell lines from the active fibrotic lesions from patients with diffuse SSc were treated with 3DG for 24 h before harvesting for RNA. cDNA transcripts of COL1A1, COL3A1, TGF-beta1, alpha1-, alpha2-, alpha5-, beta1-, and beta3-ITG, focal adhesion kinase (FAK) and paxillin genes were measured. Phosphorylation of p44/p42 MAP kinase and activity of Sp1, c-myc, and c-fos transcription factors were also measured.

Results: 3DG significantly decreased ECM expression in SSc and control fibroblasts: COL1A1, COL3A1, TGF-beta1, FAK, beta1-, alpha2- and alpha5-ITG ($P < 0.05$ for all transcripts), whereas beta3- and alpha1-ITG was increased ($P < 0.05$). We also found that phosphorylation of p44/p42 MAPK and transcription factors Sp1, c-myc, and c-fos were decreased. We further observed the disruption of signaling within the fibroblast and found that FAK and paxillin was localized to the perinuclear region of the cell. The inhibitor of 3DG, Dyn15, abolished the effects presented above and was further found to increase wound closure in an in vitro model.

Conclusions: We demonstrate that 3DG can be utilized to decrease the expression of pertinent ECM proteins and the transcription factors that control ECM over expression in SSc fibroblasts through the p44/p42 MAP kinase pathway. The 3DG inhibitor, Dyn15, abolished these adverse effects and induced a faster wound closure in an in vitro wound healing model. Taken together this data is provocative and suggests a newly discovered therapeutic pathway that may be effective in controlling fibrosis in patients with SSc.

Impact Statement: The research presented here is innovative and will lead to a better understanding of the mechanism that regulates fibrosis in SSc. This research will also have an impact for wound healing as we have a compound that decreases 3DG levels and increases wound closure rate.

3-Deoxyglucosone-collagen alters human dermal fibroblast migration and adhesion: Implications for impaired wound healing in patients with diabetes

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ABSTRACT

The interaction of fibroblasts with the extracellular matrix is critical for wound healing. Advanced glycation end products (AGEs) occur through nonenzymatic glycation of long-lived proteins such as collagens. One precursor to these modifications, 3-deoxyglucosone (3DG), is elevated in patients with diabetes and contributes to the accumulation of AGEs on collagen with increasing chronological age. Because wound repair is dependent on fibroblast migration, proliferation, and expression of extracellular matrix proteins, we examined the role of 3DG-treated collagen and the subsequent response of fibroblasts to this modification. We found that fibroblasts adhere more strongly to 3DG-treated collagen and do not migrate efficiently into the wound site. We further show that 3DG-treated collagen induces perinuclear localization of focal adhesion kinase and paxillin, indicative of decreased association of these proteins with the cytoskeleton. Additionally, these cells expressed higher levels of the misfolded indicator protein growth arrest and DNA damage inducible gene 153. These data suggest that fibroblast/matrix interactions are altered as AGEs accumulate and affect focal adhesion formation. Furthermore, 3DG may be a factor mediating chronic wounds observed in patients with diabetes and in the elderly by altering the signaling within the fibroblast and inducing the misfolding of proteins.

Cutaneous wound healing is a complex process that is vitally important in restoring the dermal barrier and preventing infection. It is known that wound healing is impaired in patients with diabetes as approximately 5–8% of patients develop chronic foot ulcers due to diabetic complications.[1,2] More importantly, patients with diabetes are at an increased risk for infection and amputation, and poor wound healing plays a significant role in the increased morbidity and mortality seen in these patients.[1,2]

The deterioration of wound-healing processes seen in diabetics is thought to result from hyperglycemia. Long-term hyperglycemia induces protein cross-linking by a nonenzymatic Maillard reaction between proteins and reactive carbonyl and dicarbonyl compounds.[3,4] Degradation of glycated proteins subsequently results in the formation of the highly reactive α-dicarbonyl, 3-deoxyglucosone (3DG), which in turn forms advanced glycation end products (AGEs).[5,6] 3DG plays a role in the modification and cross-linking of long-lived proteins such as collagen. Since the degree of glycation is determined by the exposure, duration, and concentration of glucose, AGEs are found to continually and irreversibly accumulate on collagen and increase in number as a function of age and diet, resulting in a loss in elasticity and flexibility.[7] Studies have shown that collagen glycation increases during exposure to high glucose in vivo[8] and that collagen fibers become stiffened.[9] Also, it has been established that hyperglycemia-induced AGE formation impacts the vascular, renal, and neuronal tissues of diabetic patients.[1] Recently, a definitive link was established between 3DG plasma levels and the presence of diabetic complications in humans.[10] Because 3DG is a potent AGE precursor, it is important to understand the biological activities of 3DG and its role in diabetic complications such as chronic wounds. More importantly by investigating the role of 3DG better therapeutics can emerge to treat diabetic complications. One such therapeutic is meglumine, an inhibitor of the enzyme fructosamine-3-kinase, that forms 3DG. Clinical testing has shown that meglumine in conjunction with arginine, which inactivates 3DG, can improve the appearance and texture of photodamaged skin compared with placebo. Meglumine has been shown to statistically improve visual dryness of photodamaged skin.[11] Despite all the current knowledge linking diabetic complications to AGE formation, the direct response of fibroblasts to 3DG in a wound-healing setting has not been well investigated.

Wound repair is reliant on dermal fibroblast migration, proliferation, and extracellular matrix (ECM) deposition. More specifically, wound repair involves transmission of environmental signals from the ECM through cell surface receptors that connect the actin cytoskeleton to

3DG	3-Deoxyglucosone
AG	Aminoguanidine
AGE	Advanced glycation end product
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
GADD153	Growth arrest and DNA damage inducible gene 153
MFI	Mean fluorescence intensity

downstream signaling pathways. 3DG is known to crosslink collagen in such a way that it can alter cell–ECM signaling (unpublished data).[12] These cell–ECM interactions are mediated by the integrin family of cell surface receptors and on fibroblasts, the primary adhesive interactions with collagen occurs through the α1β1 and α2β1 integrins.[13] Once integrins have coupled, the cell activates the nonreceptor protein, tyrosine kinase focal adhesion kinase (FAK). FAK and paxillin make up focal adhesions, which are important for cell migration and focal adhesion turnover.[14–17] Additionally, FAK is involved in downstream signaling cascades involving ERK1/2 and JNK/mitogen-activated protein kinases. FAK can also transduce survival signals from integrins and plays a crucial role in cell cycle progression and cell death.[17,18]

Fibroblasts deficient in FAK and paxillin appear to have altered actin cytoskeletons and do not migrate efficiently.[18–22] We have previously demonstrated that 3DG-collagen caused a potent decrease in fibroblast expression of collagen types I and III, transforming growth factor-β1, and a decrease in fibroblast proliferation (unpublished data). Additionally, we demonstrated the reversal of these deleterious effects with meglumine, an inhibitor of fructosamine-3-kinase, the enzyme that forms 3DG. Because 3DG down-regulates collagen production, we sought to better understand the role that integrins and focal adhesions play during wound healing in response to the 3DG environment. Additionally, we investigated the therapeutic role of meglumine in reversing the deleterious effects of 3DG-collagen. In the current study, we further extend our observations and compare the adhesion, migration, expression, and localization of key focal adhesion proteins in fibroblasts that were cultured on native collagen or 3DG-collagen matrices. In addition, we investigate the expression of growth arrest and DNA damage inducible gene 153 (GADD153) in fibroblasts cultured on the 3DG-collagen.

METHODS

This study was approved by the Internal Review Board of Drexel University for human studies.

Collagen coating of cultured dishes

Acid extracted type I collagen (95–97% COL1A1; 3–5% COL3A1) from human skin was purchased from Stem Cell Technologies (Vancouver, BC, Canada). The collagen was diluted in phosphate-buffered saline (PBS) to a final concentration of 0.067 mg/mL and added to the tissue culture dish for 2 hours at 37 °C as described previously.[14,19,22] After incubation, the dish was washed three times with 5 mL sterile PBS to remove any nonadherent collagen. The modified collagen adsorbed on the dish surface were incubated overnight with 1 mM 3DG and/or 5 mM aminoguanidine (AG), which were both added simultaneously to the collagen. Unincorporated 3DG and AG were removed by gently washing the collagen coating three times with 5 mL sterile PBS before plating with fibroblasts.

AG was obtained from Sigma-Aldrich, St. Louis, MO. 3DG was obtained from Toronto Research Chemicals, North York, ON, Canada. Meglumine-HCl was a kind gift from Dynamis Therapeutics Inc. (Jenkintown, PA). Meglumine has been demonstrated to inhibit the amadorase enzyme, fructosamine-3-kinase, which is the key enzyme for 3DG formation.[23]

Tissue culture

Normal human dermal fibroblasts from individuals (GM05399, GM00498, GM8333) aged 5 months to 3 years old (passage 10) were purchased from the Coriel Institute (Camden, NJ), and were seeded onto native collagen or 3DG-collagen–coated dishes and cultured until 70% confluent in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum (FBS) and 1% penicillin/streptomycin. Fibroblasts were found to be 70% confluent by 24 hours. For experiments performed at 3 hours, cells that were 70% confluent were trypsinized and seeded onto the matrices at the same density.

SYBR green quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Cells were harvested and RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. To verify expression of β1 integrin and GADD153, 2.0 μg of total RNA was reverse-transcribed using Superscript-III reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Transcripts were quantified using SYBR green PCR amplification (Qiagen). All mRNA transcripts were normalized to β-actin expression. The following primers were used to detect transcripts of interest: β1 integrin—forward: 5′-CAAAGGAACAGCAGAGAAGC-3′ and β1 integrin—reverse: 5′-ATTGAGTAAGACAGG TCCATAAGG-3′; GADD153—forward: 5′-GTAGA GACGGGGTTTCACCA-3′ and GADD153—reverse: 5′-GGCACATAGGCCTTTTGAAC-3′; β-actin—forward: 5′-TTGCCGACAGGATGCAGAA-3′ and β-actin —reverse: 5′-GCCGATCCACACGGAGTACTT-3′.

Western blotting

Cells were harvested and protein was extracted using 100 μL of cell lysis buffer supplemented with 0.3% phenylmethylsulfonyl fluoride and proteinase and phosphatase inhibitors. One hundred microgram of protein from each sample was size fractionated on 10% SDS-PAGE gels (Invitrogen) for 50 minutes at 200 V. The proteins were transferred to polyvinylidene fluoride (PVDF) membrane and the membrane blocked with 5% skim milk. The PVDF was probed with an antibody directed against either β1 integrin (1 : 200), FAK (1 : 200), paxillin (1 : 200) (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (1 : 1,000), phospho-ERK1/2 (1 : 1,000), or total ERK1/2 (1 : 1,000) (Cell Signaling Technologies, Danvers, MA). The membrane was washed with TBS-Tween to remove any unbound proteins and incubated with a secondary antibody, goat anti-rabbit HRP (1 : 2,000) or rabbit anti-goat HRP (1 : 2,000) (Jackson Labs, West Grove, PA). The signal was developed with SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL).

Wound-healing assay

Wound-healing assays were performed as described previously.[14] Scratch wounds were introduced with a sterile pipette tip onto a confluent monolayer of fibroblasts cultured on modified collagen in dual microscope chamber slides. The cells were washed three times with PBS to remove damaged or detached cells and media containing 1% FBS was added. This low amount of serum allows for cell migration to be measured in the absence of proliferation. The wound area was photographed immediately after wound induction, and again at 24 and 48 hours postscratch using brightfield exposure at ×10 magnification on a Nikon eclipse 80i epi-fluorescence microscope (Mehlville, NY). The images were captured using an RT3 Color Mosaic Camera (Diagnostic Instruments, Sterling Heights, MI) and digitally stored on a dedicated computer. The distance between the edges of the wound were measured at 10 different areas from the wound edge to edge using Spot software. Experiments were repeated three times on separate occasions using different fibroblast cell lines.

For F-actin staining of filopodia extension, fibroblasts were wounded as described above, washed three times with sterile PBS, and air-dried at 4 or 24 hours postscratch. The fibroblasts were then fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained with rhodamine phalloidin (Cytoskeleton, Denver, CO). The cell preparations were counterstained with DAPI (Vector Laboratories, Burlingame, CA). Filopodia extension was measured as length of filopodia from initial wound site. Images were captured as described above at ×20 and ×40 magnification.

Cell adhesion

Adhesion of the fibroblasts was performed on 3DG-treated collagen in chamber slides. 5×10^4 cells/mL were incubated on collagen or collagen supplemented with 5 mM AG-coated surfaces with either 1 mM 3DG or PBS for 3 and 24 hours, and then rinsed briskly with PBS to remove nonadherent cells. Adherent cells were fixed with 4% paraformaldehyde and counterstained with DAPI. A minimum of 10 images from each preparation were taken and the number of DAPI-stained cells counted and expressed as percentage of nuclei per field. All images were taken at ×10 magnification. Experiments were performed on three separate occasions.

Cell adhesion inhibition

Anti-integrin antibody adhesion assays were performed using mouse monoclonal antibodies (mAbs) against integrins β1, α1, and 2 (Santa Cruz Biotechnology) according to Chen et al.[24] to determine the involvement of integrins in cell adhesion. Seventy percent confluent cells were suspended in serum-free DMEM, incubated with antibodies (1 : 20 dilution) for 30 minutes at 37 °C and then plated on chamber slides coated with collagen or 1 mM 3DG-collagen matrices for 3 or 24 hours. The chamber slides were then rinsed briskly with PBS to remove nonadherent cells. The remaining adherent cells were fixed with 4% paraformaldehyde and counterstained with DAPI. A minimum of 10 images from each preparation were taken and number of DAPI-stained cells counted and expressed as percentage of DAPI-stained positive nuclei per field. All images were taken at ×10 magnification. Experiments were performed on three separate occasions.

Immunofluorescence

Cells cultured in chamber slides for 24 hours were fixed in 4% paraformaldehyde for 10 minutes. Cells were incubated in a 1 : 50 dilution with polyclonal antibodies against paxillin, FAK, or mAb against GADD153 (Santa Cruz Biotechnology) and incubated in a humid chamber at room temperature for 60 minutes. The samples were washed three times with PBS and then stained with Cy2 or Cy3 secondary Ab (1 : 50 dilution) (Jackson Labs) in a humid chamber at room temperature for 40 minutes. Samples were washed three times with PBS and mounted with DAPI. Images were viewed with an epi-fluorescence microscope at ×40 magnification. Mean fluorescence intensity (MFI) was calculated using ImageJ. For GADD153 studies 10 images from each preparation were taken and the MFI of the nuclei of each cell was calculated. Experiments were performed on three separate occasions.

Statistical analysis

The resulting data were subjected to two-tailed paired *t*-test for statistical significance. A p value < 0.05 was considered significant.

RESULTS

3DG increases fibroblast adhesion to collagen

In order for a cell to effectively migrate, it must adhere to collagen via integrin receptors located at the cell's surface. Previous kinetic analyses showed that weakly adhesive surfaces caused the fibroblast to poorly adhere to the substratum resulting in minimal traction and cell migration. Likewise, strongly adhesive surfaces induce immobilization of the fibroblast due to disruption of the cell–substratum attachments.[25] Previous work in our laboratory showed that there is decreased proliferation of human dermal fibroblasts cultured on 1 mM 3DG-treated collagen and so all subsequent experiments use 1 mM 3DG-collagen. First, we measured the fibroblast's adhesive strength to collagen when seeded on 3DG-collagen using a jet wash adhesion assay. Figure 1 is a graphical representation of the percentage of cells binding to native collagen, 3DG-treated collagen, and AG/3DG-treated collagen at 3 and 24 hours. Fibroblasts seeded onto native collagen begin to attach within the first 3 hours, and this attachment is difficult to disturb by mechanical disruption.[14] We observed that fibroblasts seeded on 3DG-collagen also began to attach within the first 3 hours and this attachment increased after 24 hours (Figure 1). After mechanical disruption by a stream of PBS, fibroblasts cultured on 3DG-collagen for 3 hours were found to have $26 \pm 1.41\%$ more fibroblasts attached to the collagen than that which remained on the native collagen after 3 hours (Figure 1; $p < 0.03$). After 24 hours of incubation, fibroblasts seeded on 3DG-collagen further increased their adherence to the matrix compared

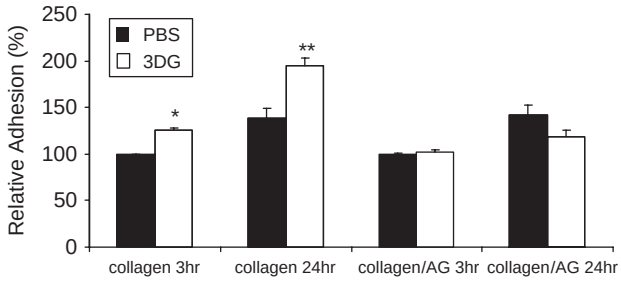

Figure 1. Effect of 3-deoxyglucosone (3DG) on fibroblast adhesion to collagen. Normal fibroblasts were seeded onto native collagen or collagen cross-linked by 1 mM 3DG and allowed to attach for either 3 or 24 hours. In some of the cross-linking experiments, aminoguanidine (AG) was added simultaneously with 3DG to chelate the 3DG. We estimated the collagen-adhesive strength by counting the cells remaining after a jet wash assay according to Arnesen and Lawson.[14] A graphical representation of the binding strength of the fibroblasts to native collagen or the 3DG/AG-collagen is presented. Cell numbers from 10 random images were counted and averaged, and presented as percentage of adhesion. This experiment was performed on three separate occasions. Fibroblasts were found to adhere $26 \pm 1.4\%$ more efficiently to the 3DG-collagen at 3 hours than the fibroblasts cultured on native collagen; $^{*}p < 0.03$. At 24 hours, fibroblasts adhered $95 \pm 8\%$ more efficiently to 3DG-collagen than the fibroblasts on native collagen; $^{**}p < 0.01$. In the presence of AG, fibroblasts were found to adhere similarly to the native collagen ($99 \pm 2.1\%$ for phosphate-buffered saline [PBS] and $102 \pm 2.1\%$ for 3DG treated at 3 hours and $42 \pm 11\%$ for PBS treated for 24 hours and $18 \pm 8\%$ for 3DG treated for 24 hours). All images were taken at $\times 10$ magnification. Comparisons from all groups are performed against collagen at 3 hours.

with fibroblasts grown on native collagen ($39 \pm 10\%$ for native collagen; and $95 \pm 8\%$ for 3DG-collagen; Figure 1; $p < 0.01$). Additional experiments with 5 mM AG, which chelates 3DG before its reaction with the collagen, did not affect fibroblast binding to the collagen and in the presence of AG, fibroblasts adhered with similar percentages to fibroblasts grown on native collagen ($99 \pm 2.12\%$ for native collagen+AG at 3 hours; $102 \pm 2.12\%$ for 3DG-collagen+AG at 3 hours; and $42 \pm 11\%$ for native collagen+AG at 24 hours; $18 \pm 8\%$ for 3DG-collagen+AG at 24 hours). This suggests that 3DG is responsible for increased fibroblast adhesion (Figure 1).

β1 integrin is essential for fibroblast adhesion to 3DG-treated collagen

Since 3DG altered the adhesion of fibroblasts to collagen, we next examined which collagen receptors were responsible for the enhanced fibroblast adhesion (Figure 2). Quantitative real-time PCR showed that fibroblasts showed increased β1 integrin expression by $22 \pm 7\%$ at 3 hours and $88 \pm 12.7\%$ at 24 hours when cultured on 3DG-treated collagen compared with native collagen (Figure 2A; $p < 0.04$ at 3 hours and $p < 0.01$ at 24 hours). When added to 3DG-treated collagen, 40 mM meglumine, an inhibitor of fructosamine-3-kinase, reduced β1 integrin transcripts below normal levels at 3 hours, expressing $48 \pm 2.82\%$ of that expressed on native collagen at 3 hours (Figure 2A; $p < 0.01$). However, at 24 hours, fibroblasts cultured on 3DG-collagen and treated with 40 mM meglumine showed normal transcript levels expressing $91 \pm 13.6\%$ of β1 integrin transcripts (Figure 2A). Western blot analysis showed that the protein expression levels of β1 integrin are also up-regulated $18 \pm 2.2\%$ at 3 hours and $65 \pm 1.41\%$ at 24 hours in response to fibroblasts cultured on 3DG-collagen (Figure 2B; $p < 0.01$).

β1 integrin is known to heterodimerize with α1 and α2 integrins, both of which are also important for cell adhesion and proliferation of fibroblasts on collagen networks.[13,26–30] We next sought to determine the functional contribution of integrins β1, α1, and α2 in 3DG-collagen binding by preincubating the fibroblasts with mAbs targeting β1, α1, or α2 integrin, and then culturing these fibroblasts on 3DG-treated collagen. Antibodies to all three integrins decreased fibroblast attachment to type 1 collagen after incubation for both 3 and 24 hours; β1 integrin induced a $54.5 \pm 2.12\%$ reduction at 3 hours and a $80 \pm 2.64\%$ reduction at 24 hours; α1 integrin induced a $26 \pm 1.41\%$ reduction at 3 hours and a $73 \pm 15\%$ reduction at 24 hours; and α2 integrin induced a $47 \pm 1.41\%$ reduction at 3 hours and a $75 \pm 3.9\%$ reduction in fibroblast binding at 24 hours (Figure 2C; $p < 0.04$ at 3 hours and $p < 0.01$ at 24 hours). With the 3DG-treated collagen, blockade of β1 integrin and α1 integrin induced a further reduction in adherence of cells to 3DG-collagen at both 3 and 24 hours of incubation: $79 \pm 2.12\%$ at 3 hours and $91 \pm 2.64\%$ at 24 hours for β1 integrin and $52 \pm 1.41\%$ at 3 hours and $89 \pm 3.68\%$ at 24 hours for α1 integrin (Figure 2C, $p < 0.04$ at 3 hours and $p < 0.005$ at 24 hours). No difference was observed by blocking α2 integrin with the 3DG-collagen at 3 or 24 hours. However, when α2 integrin binding was blocked, there was a significant decrease in the amount of adhesion observed in cells grown on either collagen or 3DG-collagen at 24 hours compared with 3 hours (Figure 2C, $p < 0.01$). These data indicate that α1β1 integrin may be more important for fibroblast adhesion to 3DG-collagen.

Fibroblast migration into the wound site is decreased in the presence of 3DG

Previous studies in our laboratory have shown that fibroblast proliferation is decreased when cultured on 3DG-collagen (unpublished data). We therefore further examined the effects of 3DG on fibroblast migration into the wound site. A scratch wound was induced by scraping a monolayer of fibroblasts that were cultured on native collagen or on 3DG-treated collagen. After the initial scratch, 3DG-collagen was reintroduced back to the chamber slides for 2 hours in order to assure that the scratch wound still contained 3DG-collagen. The media was replaced and contained 1% FBS to allow for successful migration but not the proliferation of the cells. Then the migration of fibroblasts across the introduced gap was measured at the initial time of wounding, and after 24 and 48 hours. After

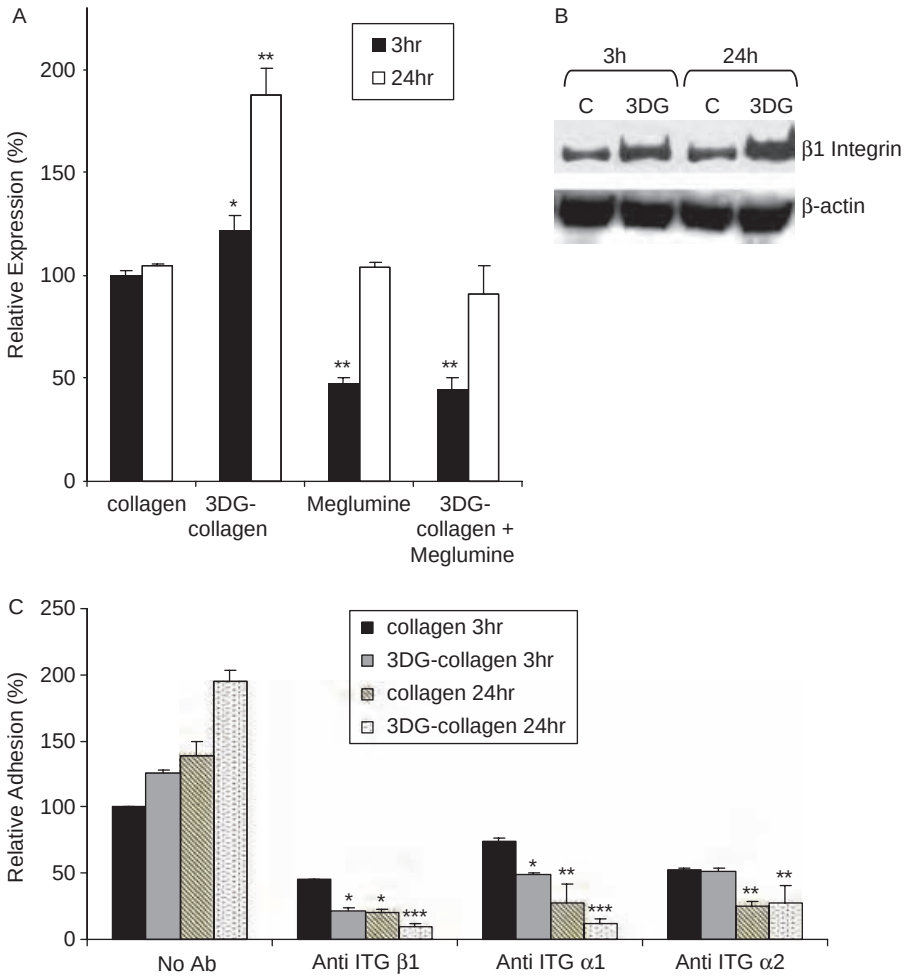

Figure 2. Involvement of β1, α1, and α2 integrins in the adhesion of fibroblasts to 3-deoxyglucosone (3DG)-collagen. Cells were seeded at a density onto matrices at 5×10^4 and cultured in Dulbecco's modified Eagle's medium (DMEM) with 1% fetal bovine serum (FBS) with or without meglumine for either 3 or 24 hours. At 3 or 24 hours, cells were lysed and RNA extracted. β1 integrin transcripts were measured by real-time polymerase chain reaction (PCR) and normalized to β-actin. (A) Expression of β1 integrin mRNA transcripts from fibroblasts cultured on native collagen, 3DG-collagen, collagen supplemented with meglumine, or 3DG-collagen supplemented with meglumine. By 3 hours, 3DG-collagen was found to increase β1 integrin expression by $22\pm7\%$ and this increased to $88\pm12.7\%$ at 24 hours compared with the fibroblasts cultured on native collagen- or the meglumine-treated cells. (B) Western blot of β1 integrin protein from fibroblasts cultured on native collagen or 3DG-collagen for 3 or 24 hours. At 3 hours, β1 integrin protein was up-regulated by $18\pm2.2\%$, which increased to $65\pm1.41\%$ at 24 hours. (C) Graphical representation of adhesion studies that were performed as described in Figure 1; however, monoclonal antibodies were used to block β1, α1, or α2 integrin-mediated binding. Inhibition of β1 integrin induced a $79\pm2.12\%$ decrease in adhesion of fibroblasts to the 3DG-collagen at 3 hours, while at 24 hours, β1 integrin blocked $91\pm2.64\%$ of fibroblast adhesion to 3DG-collagen. Data are mean ± SD (n=3); *$p<0.04$, **$p<0.01$, ***$p<0.005$. Comparisons within each group are performed against collagen at 3 hours. C, collagen; 3DG, 3DG-collagen.

24 hours, the fibroblasts cultured on native collagen showed approximately $75\pm2.08\%$ wound closure when compared with fibroblasts cultured onto 3DG-collagen ($42\pm7.4\%$, $p<0.05$). After 48 hours, fibroblasts cultured on native collagen had closed the wound by $94\pm2.96\%$, whereas the fibroblasts cultured on the 3DG-collagen had closed the wound by $68\pm3.48\%$ ($p<0.02$; Figure 3). Additionally, we supplemented the 3DG-collagen with 5 mM AG at the same time as 3DG treatment. This supplementation only partially restored the migration of fibroblasts into the wound site to $83\pm2.4\%$ at 48 hours ($p<0.05$). However, meglumine had fully closed the wound by $100\pm0.66\%$ by 48 hours (Figure 3; $p<0.02$).

When we examined the filopodia we found that 3DG decreased filopodia extension (Figure 4). Histological examination of the fibroblasts cultured on 3DG-collagen identified that these fibroblasts did not show any extension of their filopodia into the wound site at 4 hours post-scratch, whereas fibroblasts cultured on native collagen did (Figure 4A–C). The length of the filopodia was found to be $6.25\pm4.049\,\mu M$ in fibroblasts cultured on 3DG-collagen and $79\pm3.674\,\mu M$ in fibroblasts cultured on native collagen (Figure 4A–C; $p<0.0004$). At 24 hours this difference was still apparent: filopodia length was $68\pm11.098\,\mu M$ in fibroblasts cultured on 3DG-collagen and $192\pm7.348\,\mu M$ in fibroblasts cultured on native collagen (Figure 4A, D, and E; $p<0.002$).

Key focal adhesion proteins, FAK and paxillin, localize to the perinuclear region in fibroblasts adhering to 3DG-treated collagen

We have demonstrated in Figure 1 that fibroblasts cultured on 3DG-treated collagen have increased adhesion at 24 hours; however, the fibroblasts had reduced migration (Figure 3). Therefore, we examined the organization of the actin cytoskeleton and proteins associated with

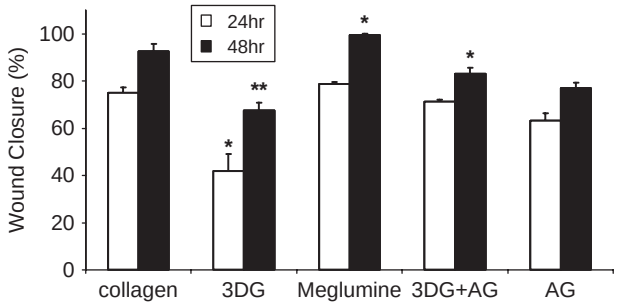

Figure 3. 3-Deoxyglucosone (3DG) slows the fibroblast migration into wound site. Confluent cultures of fibroblasts grown on 3DG-collagen were scratched manually with a pipette tip according to Arnesen and Lawson.[14] The culture media was supplemented with 3DG-collagen for 2 hours to reintroduce collagen back into the initial gap, and the scratch wound was allowed to close. After wounding, cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM 3DG-collagen, 40 mM meglumine, 1 mM 3DG-collagen/5 mM aminoguanidine (AG), or 5 mM AG. Cell migration into the wound was monitored at 24 and 48 hours by bright field visualization on an epifluorescence microscope. The distance across the wound margin was measured at 10 different points using Spot software and plotted as percentage of wound closure when compared with initial scratch at 0 hour. Fibroblasts treated with 3DG had a drastically slower wound closure rate. At 24 hours the scratch wound had closed to $42 \pm 7.4\%$, $*p < 0.05$, and at 48 hours it had closed to $67 \pm 3.48\%$, $**p < 0.02$. Meglumine, an inhibitor of 3DG, increased the wound closure rate, and at 48 hours the scratch was closed by $99 \pm 0.66\%$. Cells treated with 3DG and AG together closed the wound by $83 \pm 2.3\%$ after 48 hours. Graphical representation of the rate of the wound closure with 3DG, 3DG/AG, or the 3DG inhibitor meglumine is shown below. Data are mean $\pm$ SEM (n=3); $*p < 0.05$, $**p < 0.02$. Comparisons are performed within each time point and compared with that of collagen.

focal adhesions. FAK and paxillin are two major proteins associated with focal adhesion formation and turnover and these proteins have been shown to play a significant role in fibroblast migration.[14–16] Fibroblasts cultured on 3DG-collagen for 24 hours expressed $48 \pm 0.212\%$ less FAK protein compared with fibroblasts grown on native collagen (Figure 5A, $p < 0.002$), whereas paxillin expression did not significantly alter (Figure 5A). It is known that FAK and paxillin are normally expressed at the cell surface of migratory cells.[14,17] Because exposure to 3DG-treated collagen lowered fibroblast migratory capacity and decreased FAK expression, we sought to determine the intracellular localization of FAK and paxillin. We observed cell surface localization of FAK and paxillin in fibroblasts cultured on native collagen (Figure 5B and E). However, both these proteins were found to localize to the perinuclear region in fibroblasts cultured on 3DG-collagen (Figure 5C and F). The effects of FAK and paxillin intracellular localization in fibroblasts cultured on 3DG-treated collagen and treated with meglumine were found to be at the cell surface (Figure 5D and G).

3DG-collagen decreases fibroblast ERK1/2 phosphorylation

Phosphorylation of ERK1/2 is important in intracellular signaling and plays a crucial role in promoting cell proliferation and differentiation.[31–33] The ERK proteins are found at sites in the signaling cascades where the signals converge and activate ERK, which can regulate cytoskeletal remodeling, cell migration, and promote cell cycle progression.[34–36] It has been reported that inhibition of FAK signaling blocks MAP kinase activation in response to integrin-dependent signaling in epithelial cells.[18,19] Because 3DG-collagen induced a decrease in the expression of FAK in fibroblasts, we investigated its effect on ERK1/2 phosphorylation in fibroblasts cultured on 3DG-collagen and native collagen for 24 hours. Western blot analysis revealed that fibroblasts cultured on 3DG-collagen had a $55 \pm 2.12\%$ decrease in the phosphorylation of ERK1/2 ($p < 0.007$) compared with fibroblasts cultured on native collagen (Figure 6).

3DG increased the expression of GADD153 in fibroblasts

The presence of FAK and paxillin in the perinuclear region suggests that these proteins are being retained within the endoplasmic reticulum (ER) possibly due to protein misfolding.[14] The characteristic marker for protein misfolding and ER stress is GADD153.[37] ER stress can occur due to the accumulation of membrane-bound proteins in the ER, which in turn activates the transcription factor GADD153.[37] Under nonstress conditions, cells ubiquitously express GADD153 at very low levels in the cytosol; however, during times of cellular stress, GADD153 is induced and accumulates in the nucleus.[37] We hypothesized that the high levels of FAK and paxillin present in the perinuclear region of fibroblasts cultured on 3DG-collagen was due to improper protein folding. Transcript levels of GADD153 in fibroblasts cultured on 3DG-treated collagen was found to be increased by $83 \pm 3.9\%$, $p < 0.03$ (Figure 7A), whereas meglumine reduced GADD153 transcripts to $58 \pm 0.98\%$ that of control levels ($p < 0.03$; Figure 7A). Additionally, GADD153 transcripts remained low when meglumine was added to fibroblasts cultured on 3DG-treated collagen and was found to be $58 \pm 18\%$ of control levels (Figure 7A). Confirming this, we calculated the MFI from the histological analyses of fibroblast nuclei stained with DAPI. Histological analyses showed that cells cultured on 3DG-collagen matrices had significant localization of GADD153 in the nucleus (66.3 ± 6.641 MFI) compared with fibroblasts cultured on native collagen (43.7 ± 4.307 MFI; $p < 0.0004$; Figure 7C–D). Also, cells cultured on native collagen and treated with meglumine had significantly lower amounts of GADD153 in the nucleus (35.3 ± 4.33 MFI; $p < 0.03$; Figure 7E). To further confirm the role of 3DG in the induction of GADD153, it was noted that very low amounts of GADD153 were observed to shuttle into the nucleus in fibroblasts cultured on 3DG-collagen treated with meglumine (36.8 ± 0.59 MFI; $p < 0.0001$; Figure 7F).

DISCUSSION

With the aging and diabetic population growing in number and percentage, the need to understand the underlying

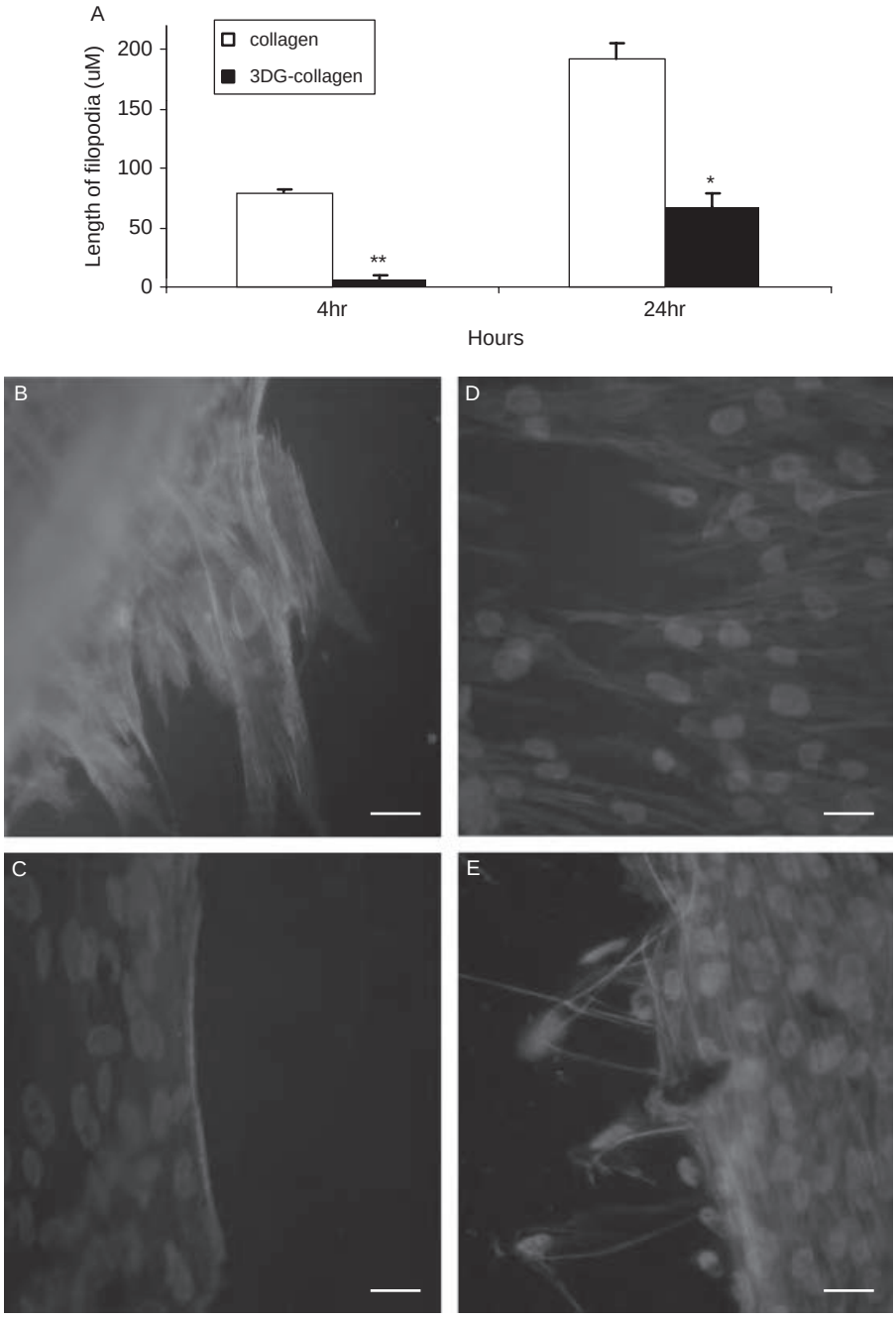

Figure 4. 3-Deoxyglucosone (3DG) decreases filopodia extension into wound site (A). Graphical representation of the filopodia extension by the fibroblast into the scratch site at 4 and 24 hours postscratch measured from (B) to (E). Filopodia extension was measured using Spot software. Extension was denoted as filopodia protrusion from initial wound site. The dotted line denotes initial wound site. All images were taken at ×40 magnification on an epifluorescence microscope with an exposure time of 2.2 seconds. On the 3DG-collagen the fibroblasts had limited extension of the filopodia compared with the native collagen, $p < 0.0004$. At 24 hours fibroblasts had started to migrate into the scratch wound, $p < 0.002$. (A–E) Fibroblasts were stained for F-actin with rhodamine phalloidin. (B, D) Fibroblasts cultured on collagen had begun to migrate into wound at 4 hours (B) and 24 hours (D) postscratch. (C, E) Fibroblasts cultured on 3DG-collagen show migration only after 24 hours (E). Data are mean ± SEM (n=3); *$p < 0.002$, **$p < 0.0004$. Scale bar represents 10 μm.

mechanisms of diabetic-related complications in wound healing has increased. 3DG has been implicated in diabetic complications, and previous work in our laboratory has shown that 3DG decreased collagen production and fibroblast proliferation.[12] These data lead us to hypothesize that the cytoskeletal organization in fibroblasts cultured on 3DG-collagen was impaired. Previously, it was shown that the organization of the actin cytoskeleton is a good indicator of the fibroblast's motility[17]; therefore, in the current study, we examined the effect of 3DG-collagen on fibroblast adhesion, migration, focal adhesion protein localization, and GADD153 induction.

We demonstrate that fibroblasts adhere more strongly to 3DG-collagen than they do to native collagen and that this increased binding was specific for 3DG (Figure 1). Fibroblasts adhered stronger to 3DG-collagen at 3 hours and increased their adhesion by 24 hours indicating that 3DG-collagen continues to affect fibroblast adherence.

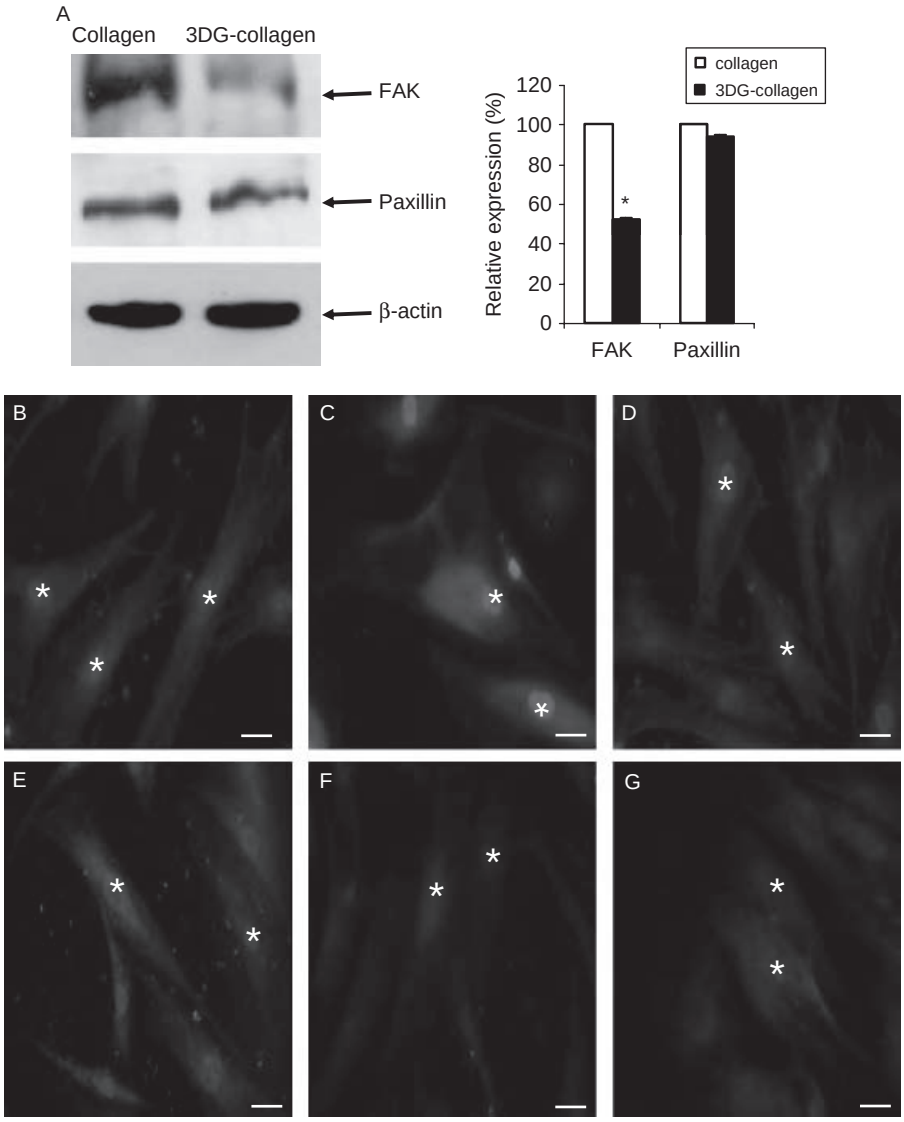

Figure 5. 3-Deoxyglucosone (3DG)-collagen matrices decrease the expression of focal adhesion kinase (FAK) and paxillin and induce localization of these proteins to the perinuclear region. The localization and expression of FAK and paxillin was measured in fibroblasts cultured on native collagen and 3DG-collagen for 24 hours. (A) Western blot of FAK and paxillin expression in fibroblasts showing decreased levels of FAK ($52.3 \pm 0.212\%$, $*p < 0.002$) in fibroblasts cultured on 3DG-collagen compared with native collagen (100%). (B–G) Immunofluorescence of focal adhesion proteins FAK (B–D) and paxillin (E–G) in subconfluent fibroblasts cultured on native collagen (B and E), 3DG-collagen (C and F), and meglumine+3DG-collagen (D and G). Localization of FAK and paxillin in the fibroblasts cultured on 3DG-collagen was found to be perinuclear. Localization was reversed when fibroblasts cultured on 3DG-collagen were treated with meglumine. All images were taken at ×40 magnification on an epi-fluorescence microscope. These data are the average of three experiments. Data are mean ± SD ($n=3$). Scale bar represents 10 μm; *nuclei.

This increased adhesion may be the reason for the decrease in cell spreading and migration. Furthermore, we found that this adherence was dependent on integrin β1. Confluent dermal fibroblasts are known to act differently than those fibroblasts in a wound. In order to mimic a wound setting in vitro, we used 70% subconfluent fibroblasts that were cultured on varying collagen-coated dishes. This subconfluent culturing allowed for subsequent qRT-PCR and Western analysis. Integrin β1 mRNA transcript levels and protein levels were increased in fibroblasts cultured on 3DG-treated collagen, which led us to further show that fibroblasts adhered more strongly to 3DG-treated collagen via β1 integrin (Figure 2A and C). β1 integrin is a receptor that is essential for cellular adhesion to a variety of ECM proteins, in particular, collagen.[24,38] In order to elicit binding to collagen, the β1 integrin subunit must pair up with one of several α subunits.[13,29] In the present study, we show that α1 integrin may heterodimerize with β1 integrin for maximal binding of the fibroblast to 3DG-treated collagen (Figure 2B).

The strong adherence between the fibroblast and the 3DG-collagen led to a shift in the dynamics of fibroblast mobility induced by the stronger adherence of the fibroblast to the collagen molecule leading to immobilization of the cell. This we confirmed with the scratch assay. By utilizing the scratch assay, we were able to demonstrate that 3DG-collagen mediated a reduction in the wound closure rate (Figure 3). In addition, the filopodia in fibroblasts cultured on 3DG-collagen did not begin to extend into the wound margin until 24 hours post-scratch. This observation was dramatically delayed when compared with fibroblasts cultured on native collagen,

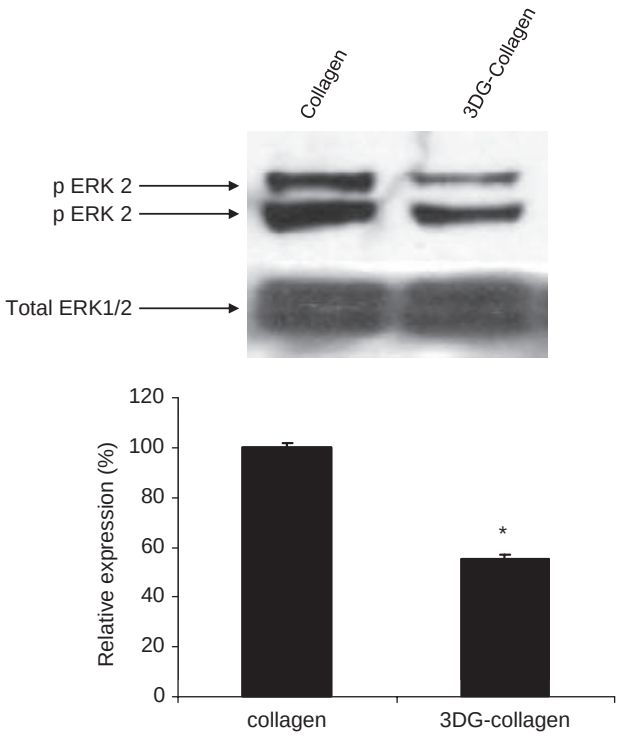

Figure 6. 3-Deoxyglucosone (3DG)-collagen decreased ERK1/2 phosphorylation. Western blot showing decreased protein levels of phosphorylated ERK1/2 in fibroblasts cultured on 3DG-collagen ($55 \pm 2.12\%$, $*p < 0.007$) compared with fibroblasts cultured on native collagen for 24 hours. Total ERK1/2 was used as a protein loading control. These data are the average of three experiments. Data are mean $\pm$ SD (n=3).

which had begun to extend their filopodia within 4 hours (Figure 4A–E).

The significant decrease in fibroblast migration led us to investigate focal adhesion proteins. Focal adhesions are not only required to transmit signals from the ECM to the cell for proliferation, but they are also required for cellular traction that is necessary for migration.[19] Therefore, altered expression or localization of these proteins could lead to a cell's inability to migrate efficiently. While paxillin expression was not significantly altered, FAK was significantly decreased in fibroblasts cultured on 3DG-collagen (Figure 5A). More importantly, the localization of both FAK and paxillin was altered and these proteins were identified in the perinuclear region, not at the cell surface (Figure 5B–G). FAK and paxillin are localized to the cytoplasm and can be found immediately below the cell's surface; however, they normally do not enter the ER or Golgi network.[15–17] One explanation for this occurrence could be a high degree of misfolded proteins present in the fibroblasts cultured on the 3DG-collagen. It has been previously demonstrated that when misfolded proteins are not degraded, they can form aggregates around the microtubule-organizing center, which is situated in close proximity to the nucleus.[39] FAK degradation can lead to decreased MAPK activation, which in turn could lead to decreased cell spreading and proliferation.[17] We also demonstrate that 3DG depressed ERK1/2 phosphorylation, which is also known to decrease cell proliferation (Figure 6). These data show 3DG can impact FAK expression, which can then elicit deleterious effects on the cells' mobility and proliferation.

Because the focal adhesion proteins were localized in the perinuclear region, this led us to investigate GADD153, which is a marker for misfolded proteins in the ER. We found that GADD153 transcript levels increased 83% in fibroblasts cultured on 3DG-collagen and this was apparent by the high amounts of GADD153 shuttling into the nucleus (Figure 7). Additionally, the use of meglumine inhibited GADD153 expression. The inhibition of GADD153 by meglumine showed that the induction of GADD153 in this system was 3DG-dependent. Previous work in our laboratory has shown that 3DG induced caspase-3, a marker for apoptosis (unpublished data). It has been well documented that ER stress can induce GADD153, which leads to apoptosis of the cell through an incompletely understood mechanism.[37,38] These data suggest that 3DG-treated collagen may be inducing ER stress resulting in caspase-3 activation.

This current study has established that in vitro exposure of human fibroblasts to 3DG-treated collagen leads to a reduction of key focal adhesion proteins and up-regulation of GADD153. This inhibition and relocalization of focal adhesion proteins could be due to the highly dynamic interaction between integrins and the 3DG-collagen, which induced immobilization of fibroblasts and reduced cell migration by the stronger adherence of the fibroblast to the collagen. We propose that 3DG-induced changes in fibroblasts can induce an increase in the expression of collagen-specific β1 integrin, leading to the increased adherence and decreased migration of fibroblasts. Indeed, misfolding of focal adhesion proteins could cause both up-regulation of GADD153, and down-regulation of ERK1/2, ultimately leading to decreased cell proliferation and spreading. Fibroblasts cultured with meglumine, which is an inhibitor of 3DG formation, resulted in increased scratch wound closure and decreased GADD153. This observation supports the role of 3DG in aberrant fibroblasts.

As a significant number of diabetics have chronic ulcers (5–8% of the diabetic population)[1] and chronic wounds in the elderly comprise approximately 8% of the population (excluding diabetic wounds),[39] it is important to understand the role of AGE-collagen modification in fibroblast signaling. By understanding the relationship between collagen and the fibroblast, we can identify better therapies for patients with poor wound-healing properties. This study showed the deleterious effects of 3DG-collagen on fibroblast focal adhesion protein expression and localization, induction of GADD153, and on fibroblast migration. These results provide a foundation for the signaling elicited between the fibroblast and the 3DG-treated collagen and may yield clues to the poor wound-healing capacity observed in patients with diabetes. By elucidating this signaling pathway, new therapeutics may become available for the increasing population suffering from chronic wounds.

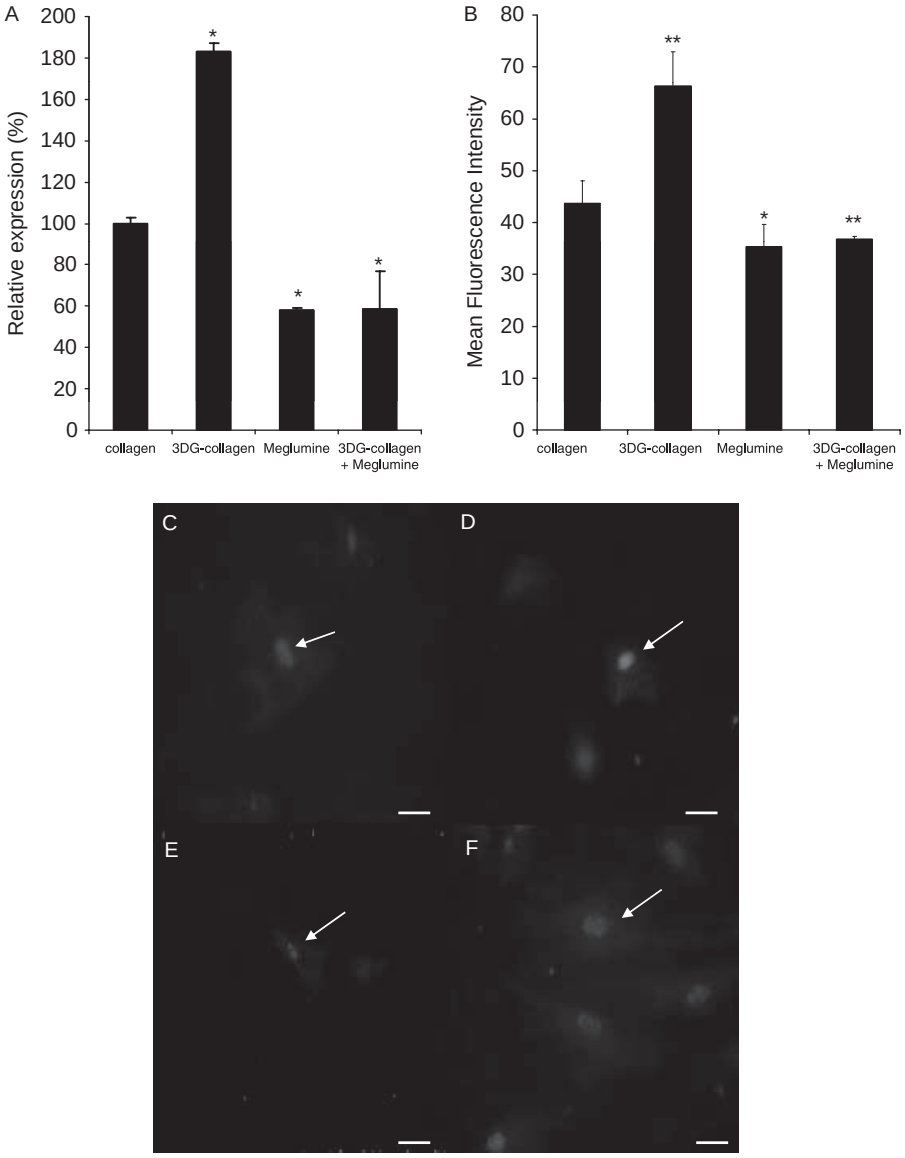

Figure 7. 3-Deoxyglucosone (3DG)-collagen increased growth arrest and DNA damage inducible gene 153 (GADD153) expression and induced its localization to the nucleus. (A) Normal fibroblasts were cultured on native collagen, 3DG-collagen, or treated with 40 mM meglumine until 70% confluent. RNA was extracted and GADD153 transcripts were measured by real-time polymerase chain reaction (PCR). Transcripts were normalized to β-actin. 3DG-collagen was found to increase GADD153 levels by $83 \pm 3.9\%$, $*p < 0.03$, whereas meglumine decreased GADD153 levels by $42 \pm 18\%$, $*p < 0.03$. (B) Graphical representation of the immunofluorescence of GADD153 in the nucleus of fibroblasts cultured on collagen, 3DG-collagen, or with meglumine measured with ImageJ from representative fibroblasts (C–F). Fibroblasts cultured on 3DG-collagen showed increased levels of GADD153 in the nucleus (66.3 ± 6.641 MFI) compared with control (43.7 ± 4.307 MFI). This was statistically significant: $**p < 0.0004$. Meglumine was found to decrease GADD153 to 35.3 ± 4.33 MFI. Fibroblasts cultured on 3DG-collagen and treated with meglumine showed reduced levels of GADD153 in the nucleus (36 ± 0.55). This was statistically significant: $***p < 0.0001$. All images were taken at ×40 magnification. Data are mean ± SD (n=3); $***p < 0.0001$, $**p < 0.0004$, $*p < 0.03$. Scale bar represents 10 μm.

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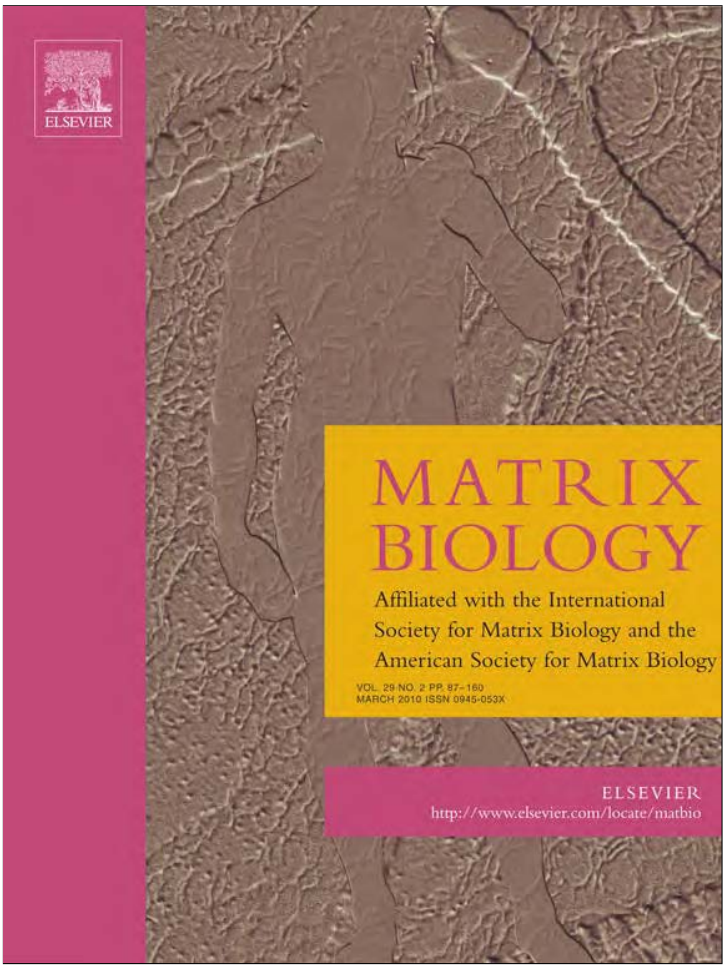

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Two dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts

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ABSTRACT

Advanced glycation endproducts accumulate on long-lived proteins such as collagens as a function of diet and age and mediate the cross-linking of those proteins causing changes in collagen pathophysiology resulting in the disruption of normal collagen matrix remodeling. Two commonly studied advanced glycation endproduct precursors 3-deoxyglucosone and methylglyoxal were investigated for their role in the modification of collagen and on extracellular matrix expression. Fibroblasts cultured on methylglyoxal cross-linked matrices increased the expression of collagen, active TGF-beta1, beta1-integrin, and decreased Smad7; whereas 3-deoxyglucosone decreased collagen, active TGF-beta1, beta1-integrin but increased Smad7. Purified collagen modified by 3-deoxyglucosone or methylglyoxal had different molecular weights; methylglyoxal increased the apparent molecular weight by approximately 20 kDa, whereas 3-deoxyglucosone did not. The differences in collagen expression by 3-deoxyglucosone and methylglyoxal raise the provocative idea that a genetic or environmental background leading to the predominance of one of these advanced glycation endproduct precursors may precipitate a fibrotic or chronic wound in susceptible individuals, particularly in the diabetic.

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1. Introduction

Fibroblasts are responsive to external signals from the extracellular matrix (ECM) and the interaction of fibroblasts with the ECM is essential in many physiological and pathological processes (Mauch and Krieg, 1990; Eckes et al., 2000). The ECM provides the three-dimensional structure that is required for fibroblast cell adhesion and migration (Postlethwaite et al., 1987; Clark et al., 2003; Kessler et al., 2001). It was originally thought that fibroblasts only produced ECM proteins for the structural integrity of the organ; however, it is now recognized that fibroblasts are capable of synthesizing many soluble molecules including growth factors, cytokines, and lipid mediators that are also able to influence cell–matrix, and cell–cell interactions (Kessler-Becker et al., 2004).

Fibroblasts are sensitive to the mechanical tension in the environment that surrounds them and alteration in ECM mechanical stresses are potentially due to the accumulation of advanced glycation endproducts (AGEs) that modulate signaling within the fibroblast altering the balance in metabolism of the ECM. Glycation of long-lived proteins such as collagen increases with age and diet (Schnider and Kohn, 1981). Type I collagen (COL1A1) is the most abundant collagen in the skin and was the first ECM protein to be shown to be covalently cross-linked by AGEs (Kent et al., 1985), resulting in increased stiffness comparable to changes seen in diabetic patients (Reihsner and Menzel, 1998; Reihsner et al., 2000). Indeed, glycation was found to alter the structure of the collagen fiber by increasing the expansion of collagen through intermolecular cross-linking (Tanaka et al., 1988). Physiological glycation can involve the modification of collagens by reactive α-oxoaldehydes, especially 3-deoxyglucosone (3DG) and methylglyoxal (MG). MG has been reported to inhibit the binding of COL1A1 to the α2β1 integrin receptor on fibroblasts. This, in turn, inhibited collagen phagocytosis suggesting that MG may be involved in some pathologic fibrotic conditions (Chong et al., 2007). In contrast, we report that α1β1 integrin binds 3DG-collagen more strongly to fibroblasts than native collagen, that 3DG-collagen inhibited filopodia extension into scratch wounds, and induced localization of focal adhesion kinase and paxillin away from focal adhesions to the perinuclear region (Loughlin and Artlett, 2009).

The notion that mechanical tension generated by AGE cross-linking of collagens and translating this external tension into fibroblast ECM protein expression is provocative but not well understood. It may be an important pathological feature in some fibrotic conditions, such as scleredema diabeticorum and in chronic wounds. Diabetic patients frequently have wound healing problems and yet a proportion of these

Abbreviations: 3DG, 3-deoxyglucosone; AG, aminoguanidine; AGE, advanced glycation endproducts; COL1A1, type 1 collagen; COL3A1, type 3 collagen; ECM, extracellular matrix; MF, morpholinofructose; MG, methylglyoxal.

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patients have scleredema diabeticorum, which is a distinct cutaneous manifestation of thickened skin generally occurring on the posterior of the neck and upper back. Scleredema diabeticorum occurs in approximately 2.5% of the diabetic population (Cole et al., 1983) and diabetic foot ulcers occur in approximately 5% of patients (Abbott et al., 2002). Both of these diabetic features appear to be a pathological dichotomy and yet they are associated with poor glycemic control. Understanding how different AGE precursors affects collagen expression via cross-linking of collagen matrices will yield important clues to these pathological conditions, particularly in diabetes.

When we studied 3DG; we observed that fibroblasts cultured with this AGE precursor decreased the expression of collagen. This observation was surprising and intriguing as previous studies, primarily with MG, reported an increase in collagen expression (Chong et al., 2007; Golej et al., 1998; Paul and Bailey, 1999). Therefore, this study was designed to explore some of the differences observed between 3DG and MG and to gain a better understanding of the role of different AGE precursors and their effects on the fibroblast. Thus, we investigated the role of 3DG and MG, on the in vitro cross-linking of collagen molecules and the subsequent altered collagen expression in normal cultured fibroblasts.

2. Results

2.1. 3-Deoxyglucosone and methylglyoxal modify collagen matrices differently

The α-dicarbonyl compounds 3DG and MG are reactive compounds that are capable of reacting inter-molecularly and intra-molecularly with amino groups resulting in the formation of stable AGEs. Electron microscopy analysis revealed that glycated collagen fibers had larger, irregular diameters (Bai et al., 1992) caused by the expansion of intermolecular spaces between the collagen fibrils (Tanaka et al., 1988). Therefore, we incubated collagen with 1 mM 3DG or 1 mM MG for increasing amounts of time to determine the rate of modification of the collagen molecule (Fig. 1). Collagen was then size fractionated on gels to determine if these AGE precursors affected the collagen fibrils. We found that MG treated collagen at 1 h had a size shift, with an increasing shift in apparent molecular weight with increasing time. In contrast, we saw no detectable shift in the apparent molecular weight with 3DG until 18 h and with increasing time, the shift in apparent molecular weight of the collagen did not increase beyond that which was observed at 24 h. To confirm the specificity of the AGE precursor to collagen modification, we incubated the AGE precursor with 1 mM aminoguanidine (AG) for 48 h completely inhibited the shift in size fractionation of the collagen confirming that the shift in apparent molecular weight was due to the AGE precursor. As 3DG exhibited a slower rate of collagen modification, we also treated collagen with 1 mM 3DG for 6 days and observed no further size shift in collagen than that which was observed at 48 h (data not shown). This data suggests that the modification of collagen by 3DG is different from the modification modulated by MG.

2.2. Morpholinofructose induces 3-deoxyglucosone in fibroblasts and concordantly decreases hydroxyproline (total collagen)

Many types of cells are able to utilize morpholinofructose (MF), which is an alternative substrate for the enzyme fructosamine 3-kinase and results in increased levels of 3DG. We investigated the production of 3DG by confluent dermal fibroblasts cultured with MF. Two 70 mm dishes each from the 9 different fibroblast cells lines were cultured at low passage (passage 2–4), with or without 10 mM MF for 24 h. 3DG liberated into the media was extracted and measured by GC-MS. Fibroblasts cultured without MF liberated 780 (+/−213) μmol/l of 3DG compared to 1893 (+/−161) μmol/l with MF; ($P<0.0001$). Likewise, intracellular 3DG levels in fibroblasts were found to be elevated; 8.3 (+/−3.0) μmol/l 3DG in the controls vs. 27.9 (+/−6.7) μmol/l 3DG with MF, ($P<0.0001$). Five of the primary fibroblast cell lines cultured with MF were assessed for hydroxyproline (total collagen) and were found to express less collagen than the fibroblasts cultured without MF. Fibroblasts cultured with 10 mM MF had an average hydroxyproline expression of 63.6% of that expressed by the control ($P=0.0032$). The decrease in the expression of COL1A1 and COL3A1 was confirmed at the mRNA level. Fibroblasts cultured with MF also had a corresponding decrease in mRNA of COL1A1; 62%, $P=0.0002$ and COL3A1; 39%, $P<0.0001$ compared to the control.

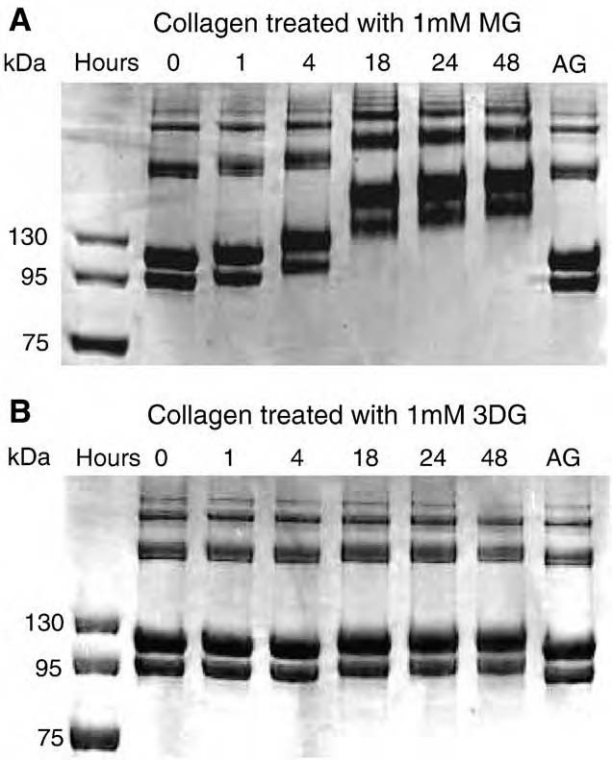

Fig. 1. Rate of modification of collagen by 3DG and MG. Normal human collagen was incubated for either 1 mM MG (Panel A) or 1 mM 3DG (Panel B) at 37 °C for increasing amounts of time: 1, 4, 18, 24, and 48 h. In addition, we incubated collagen with the AGE precursor for 48 h supplemented with 5 mM aminoguanidine (AG). The protein was size fractionated on a 10% SDS denaturing gel and stained with Coomassie for 60 min and destained prior to scanning.

2.3. 3-Deoxyglucosone and methylglyoxal cross-linked collagen matrices modulate COL1A1 and COL3A1 expression differently

Collagen matrices were treated with 1 mM, 2 mM or 4 mM 3DG; or 0.01 nM, 0.1 nM, or 1 nM MG overnight and normal fibroblast cell lines were cultured on the modified matrices for 3 days. RNA was extracted and COL1A1 and COL3A1 mRNA transcripts were quantified by SYBR Green real time PCR. With the MG treated collagen matrices, fibroblasts increased the expression of both COL1A1 and COL3A1 mRNA (data not shown). When we analyzed the same titrations of MG and measured COL1A1 and COL3A1 protein by Western blotting, we found that COL3A1 expression was more sensitive to the presence of the MG modification, demonstrating approximately 3-fold increase in COL3A1 at the highest concentration (Fig. 2). However, COL1A1 protein did not increase as significantly with MG. In contrast, fibroblasts cultured on the 3DG collagen matrices consistently demonstrated a decrease in expression of COL1A1 and COL3A1 mRNA transcripts (data not shown) with a corresponding decrease in protein (Fig. 2). Furthermore, in contrast to MG collagen matrices, we observed that COL1A1 was more sensitive to 3DG, demonstrating a larger decrease in protein expression than that demonstrated by COL3A1 (Fig. 2).

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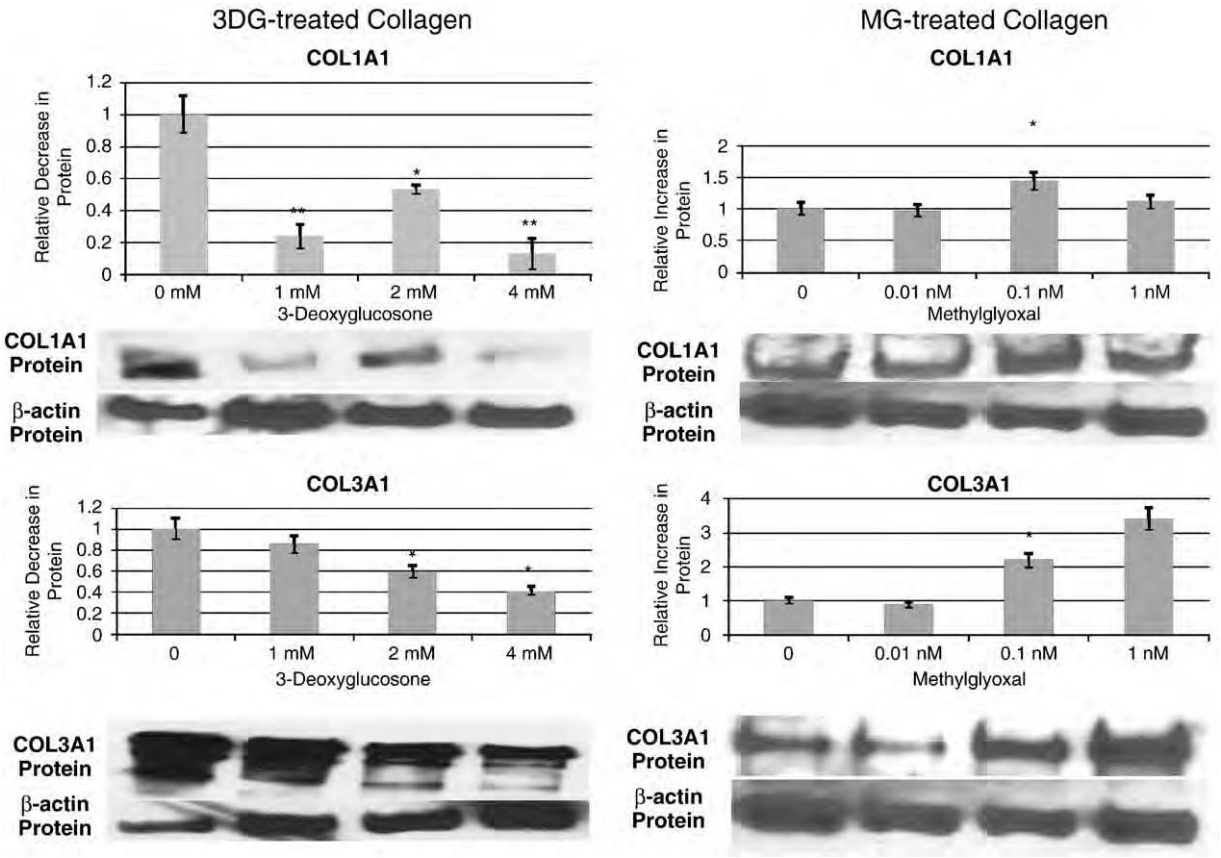

Fig. 2. Expression of COL1A1 and COL3A1 protein in fibroblasts cultured on collagen matrices modified by 3DG or MG. Fibroblasts cultured on collagen matrices cross-linked with increasing amounts of 3DG (0, 1, 2 or 4 mM) or MG (0, 0.01, 0.1 or 1 nM) for 24 h were assayed for COL1A1 and COL3A1 protein by Western blotting. Bands were quantified by ImageJ and plotted relative to the concentration found in the untreated fibroblasts. We demonstrate that COL1A1 and COL3A1 protein was decreased with increasing 3DG cross-linking of the collagen matrix, whereas COL1A1 and COL3A1 mRNA and protein was increased with MG cross-linked collagen. Values are presented as mean +/− SD, *$P<0.01$; **$P<0.001$.

2.4. Transforming growth factor-β1 and β1-integrin mRNA is down regulated in response to the 3-deoxyglucosone-modified collagen matrices but upregulated with methylglyoxal-modified collagen matrices

TGF-β1 is a critical cytokine involved in the basal regulation of COL1A1, COL3A1, and β1-integrin. Upregulation of TGF-β1 is observed in fibrotic disorders and results in a co-ordinate increase in the expression of the collagen genes. Therefore, we investigated the expression of TGF-β1 in fibroblasts, using the same RNA that was used to determine the COL1A1 and COL3A1 transcripts. In keeping with the decreased expression of the collagen genes, we found TGF-β1 to be decreased with 1 mM 3DG-treated collagen matrices ($P<0.03$). We observed a slight increase the expression of TGF-β1 with 0.1 nM MG-treated collagen matrices; this was not statistically significant (Fig. 3). TGF-β1 protein was decreased in fibroblasts cultured on 1 mM 3DG-treated collagen matrices ($P<0.001$) and increased in 0.1 nM MG-treated collagen matrices ($P<0.03$; Fig. 3). We measured active TGF-β1 protein by ELISA in the culture supernatants in the same cells cultured on 3DG or MG treated collagen. Fibroblasts cultured on native collagen secreted into the media 98.3 pg/ml of TGF-β1; this was found to increase to 114.7 pg/ml (1.2 fold increase) with MG and decreased to 40.9 pg/ml (60% decrease) with 3DG ($P=0.02$).

Furthermore, in analyzing β1-integrin we found that the mRNA was also decreased in cells cultured on the 1 mM 3DG treated matrices ($P<0.001$) and increased in cells cultured on the 0.1 nM MG treated matrices ($P<0.001$). Western blot analysis confirmed that cells cultured with 1 mM 3DG decreased β1-integrin protein ($P<0.001$) whereas 0.1 nM MG increased β1-integrin protein in fibroblasts but this was not significant (Fig. 3).

2.5. Smad7 expression is increased in fibroblasts cultured on 3-deoxyglucosone-modified collagen and decreased on methylglyoxal-modified collagen

TGF-β1 signals from the TGF-β1 receptor through the Smad pathway. The Smad pathway encompasses Smad3 heterodimerizing with Smad4 inducing their translocation to the nucleus where they interact with Smad-binding elements in promoters (e.g. collagen). Smad7 interferes with this heterodimerization, thus preventing the activation of Smad sensitive promoters (reviewed in Varga, 2002). We found that Smad7 protein was induced in fibroblasts cultured on 1 mM 3DG-modified collagen matrices, whereas it was decreased in fibroblasts cultured on 0.1 nM MG-modified collagen matrices (Fig. 4).

3. Discussion

While investigating the AGE precursor 3DG, we observed that fibroblasts cultured with 3DG or MF which is metabolized to 3DG, expressed less COL1A1 and COL3A1 mRNA transcripts and protein. We found this to be intriguing. The relationship of AGEs to fibrosis is important in diseases such as diabetes; however, there are situations where chronic wounds are a pathological problem suggesting the role of different AGEs in the pathology of diabetes is important but not completely understood. For this reason, we studied MG and 3DG to determine their effect on collagen expression in dermal fibroblasts.

As fibroblasts are able to metabolize MF to 3DG, we measured the amount of 3DG release by MF by HPLC and found it to be significantly increased. More importantly, the 3DG was measured as free 3DG not

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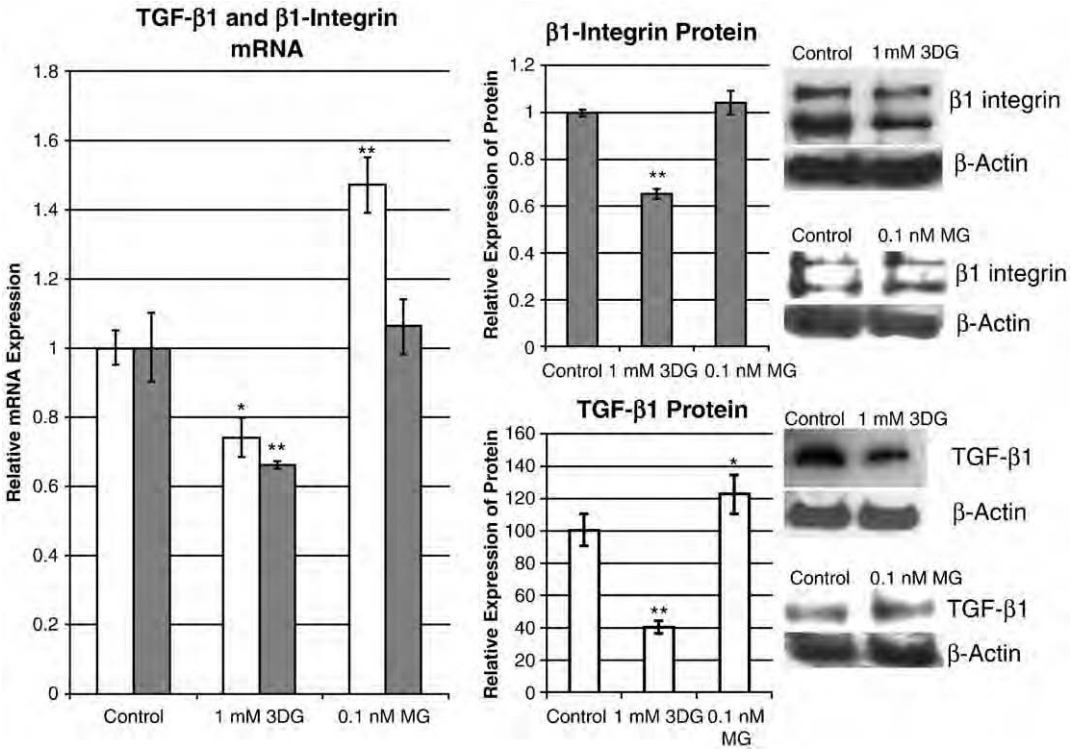

Fig. 3. TGF-β and β1-integrin expression is decreased by 3DG modified collagen matrices and increased with MG modified collagen matrices. RNA extracted from six confluent fibroblast cell lines in Fig. 2 and assayed for TGF-β1 (white bars) and β1-integrin (gray bars). Values are presented as mean +/− SD. In concordance with the decreased collagen expression with 1 mM 3DG and the increased collagen expression with 0.1 nM MG, we observed a decrease in TGF-β1 mRNA in cells cultured on 1 mM 3DG-modified collagen matrices and an increase in TGF-β1 expression in cells cultured on 0.1 nM MG-modified collagen matrices. Protein analysis demonstrated that MG and 3DG concordantly increased or decreased β1-integrin and TGF-β1, respectively. Protein concentrations were corrected for β-actin protein. 3DG = 3DG-modified collagen matrices, MG = MG-modified collagen matrices, C = native collagen with no modifications. *$P<0.03$, **$P<0.001$.

3DG chelated to proteins. The fibroblast response to MF demonstrated that fibroblasts expressed less hydroxyproline. Hydroxyproline is an uncommon amino acid that is specific for collagen and therefore we concluded that less total collagen protein was being secreted in the presence of 3DG. We also confirmed this at the RNA level and found that the mRNA of COL1A1 and COL3A1 transcripts were depressed when fibroblasts were cultured with MF.

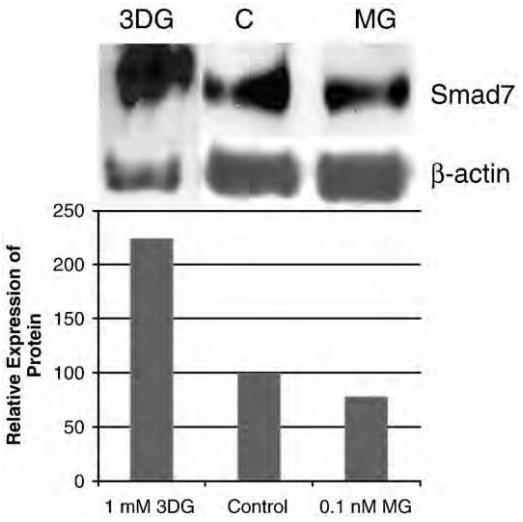

Fig. 4. Smad7 expression is increased by 3DG modified collagen matrices and increased with MG modified collagen matrices. Fibroblasts were cultured on 1 mM 3DG collagen matrices or 0.1 nM collagen matrices for 24 h. Protein was extracted from the cells and size fractionated on polyacrylamide gels as described in the methods. SMAD7 expression was elevated in the fibroblasts cultured on 3DG-collagen matrices and decreased with MG collagen matrices, resulting in the inhibition or induction of collagen expression, respectively. 3DG = 3DG-modified collagen matrices, MG = MG-modified collagen matrices, C = native collagen with no modifications.

We next examined the role of 3DG and MG (MG because it was reported to increase collagen expression), in the modulation of collagen expression by culturing normal fibroblasts on collagen matrices cross-linked with these compounds. We observed that fibroblasts cultured on 3DG-modified-collagen matrices had reduced expression of collagen protein (COL1A1 and COL3A1), whereas fibroblasts cultured on MG-modified-collagen matrices had increased COL3A1 protein (Fig. 2). Therefore, we found that COL1A1 expression was more sensitive to the 3DG-modification of the collagen molecule than COL3A1 as 3DG reduced COL1A1 expression by up to 80%, whereas COL3A1 was reduced by 15%. However, with 0.1 nM MG, COL1A1 increased 1.4 fold, but COL3A1 increased approximately 2.2-fold suggesting that COL3A1 expression was more sensitive to the MG modification. Recently, Tang et al. demonstrated that COL1A1 and COL3A1 were differentially regulated in cardiac fibroblasts when cultured with BSA-AGE. They observed that COL1A1 was increased; whereas, COL3A1 was decreased (Tang et al., 2008). This suggests COL1A1 and COL3A1 are not always tandemly regulated, or regulated at the same rate. COL1A1 and COL3A1 are located on different genes; chromosome 17 for COL1A1 and chromosome 2 for COL3A1 and although their expression is mediated by TGF-β1 this observation suggests that different signaling events are responsible for the differing amounts of collagen expression in response to MG or 3DG. Our data also confirms that of Tang et al. (2008) and also suggests that AGEs can modulate the expression of collagens differently.

The size increase observed with the treatment of collagen with MG (Fig. 1) confirms the observations by Chong et al. (2007) who demonstrated an increase in the size of collagen with MG. In our experiment we found that collagen treated with 1 mM MG was found to have an apparent increase in molecular weight, whereas collagen

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that was treated with 3DG did not increase in size as significantly, suggesting that the adduct that is formed during the cross-linking of the collagen molecule is more important than the amino acid which is altered, as both MG and 3DG alter the same amino acids (lysine and arginine). What is clear from the data is that collagen matrices modified by 3DG or MG, had profound and opposing effects on collagen expression. MG and 3DG modify the collagen at different rates; however, both AGE precursors induced detectable shifts in the apparent molecular weight of the collagen molecule by 18 h (Fig. 1). MG continued to induce a size shift in the collagen molecule with increasing time, whereas 3DG did not. Furthermore, when we incubated collagen with 1 mM 3DG for 6 days, there was no further increase in size of the collagen molecule to that which was observed at 24 h (data not shown). This observation was also confirmed by culturing fibroblasts on 3DG-collagen modified for 6 days and the decrease in collagen expression by real time PCR was still observed. Indeed, this suggests that both 3DG and MG modify collagen differently and that the alteration in collagen expression is not due to the rate of modification by these AGE precursors. Our data further confirms the recent findings of Molinari et al. (2008) who investigated the effects of AGE products on gene expression in dermal fibroblasts. AGEs alter the charge on collagen molecules (Hadley et al., 1998) and therefore may alter the interaction of collagen with its integrin binding receptor. COL1A1 contains three triplet integrin binding motifs (GFOGER, GLOGER, and GASGER) that bind the α2β1 integrin receptor with conserved spacing of these sequences along the collagen molecule (Farndale et al., 2003). MG induces an apparent increase in the size of the collagen molecule, most likely through the disruption in the spacing of the collagen fibers, which we speculate makes it inaccessible to the α2β1 integrin receptor. More recently, investigations demonstrated that MG can lead to glycation of the Arg-390, Arg-889, and Arg-1452 residues on type IV collagen and that these amino acids corresponded to the sites on collagen that interact with α1 and α2 integrins (Dobler et al., 2006). This mechanism may also be in operation for COL1A1. As MG-modified collagen was found to decrease the attachment of cells to collagen (Dobler et al., 2006; Arnesen and Lawson, 2006), we speculated that 3DG-modified collagen matrices might increase the binding of cells to collagen thus signaling the cell to turn off collagen expression. Indeed, using the jet wash assay described by Arnesen and Lawson (2006) we found that 3DG-collagen retained approximately 30% more fibroblasts on the matrix than did native collagen and that as the fibroblast was more strongly tethered to the 3DG-collagen fiber its migration was impeded in an in vitro scratch wound assay (Loughlin and Artlett, 2009). Finally we found that β1 integrin was important for binding 3DG-collagen (Loughlin and Artlett, 2009). As Dobler et al. (2006) and Arnesen and Lawson (2006) reported the decreased attachment of the fibroblast to the collagen fiber, our data further highlights that the modification to the collagen fiber with 3DG is different from that observed with MG, that the fibroblast interacts with the collagen fiber differently mediating the altered downstream signaling and gene expression.

Prior to the seeding of fibroblasts onto the 3DG- or MG-modified collagen matrices; the matrices were washed extensively to remove any unreacted 3DG or MG. As 3DG and MG are highly reactive, the possibility of un-reacted precursors associated with the collagen would be unlikely; however, we considered this step to be a necessary precaution. Indeed, we found that after washing the collagen matrix with PBS, there was a residual of 0.6 μM of free 3DG in the culture media and in our titration experiments, this would have not been enough 3DG to alter fibroblast collagen expression. These findings suggest that 3DG changed the collagen in such a way to alter cellular signaling, resulting in the decrease in both COL1A1 and COL3A1 expression.

In response to the treated matrices, intracellular TGF-β1 mRNA and protein, and active secreted TGF-β1 was decreased with 3DG but increased with MG. This finding confirms for MG the previously published data by Yamagishi et al. (2003), who studied the effects of MG-BSA on kidney epithelial cells but here we present a decreased in TGF-β1 expression with 3DG. TGF-β1 is a pleiotrophic cytokine and modulates the expression of many proteins (collagen being one of the most well studied) but it is also known to modulate the expression of β1-integrin (Zambruno et al., 1995) and therefore in response to the decrease in TGF-β1 (mRNA and protein), we observed a decrease in β1-integrin mRNA and protein with 1 mM 3DG, and a corresponding increase of β1-integrin mRNA and protein with 0.1 nM MG treated matrices (Fig. 3). Confirming the role of TGF-β1, we found that Smad7 protein was increased in fibroblasts cultured on 3DG-modified collagen and decreased in fibroblasts cultured on MG-modified collagen matrices (Fig. 4). Smad7 serves as an adaptor protein and regulates the transcription of collagen (and many other genes) in response to TGF-β1 by inhibiting the interaction of Smad3 with Smad4 (Chen et al., 1999). Thus, the translocation of Smad3/4 to the nucleus where it binds to Smad binding elements in the promoters of genes is inhibited. Therefore, finding that Smad7 was elevated in fibroblasts cultured on 3DG-modified collagen matrices along with the decrease in TGF-β1 protein suggests a direct signaling mechanism for the decreased collagen expression observed with the 3DG-matrices through the regulation of TGF-β1. We observed the opposite for MG-modified collagen matrices; increase in TGF-β1 and a decrease in Smad7 protein, therefore leading to an increase in collagen gene expression.

The findings presented here were surprising as we found that MG and 3DG modulated collagen expression differently. MG and 3DG are both elevated in diabetics and these compounds appear to correlate with diabetic complications (Beisswenger et al., 2005; Fosmark et al., 2006). Interestingly, a diabetic patient can present with either chronic ulcers or a fibrotic condition called scleroderma diabeticorum. The differences observed by our in vitro analyses may pave the way for understanding of these two opposing pathologies in diabetes. We believe a genetic background predisposes the individual to either chronic wounds or fibrosis determined by the balance between 3DG and MG. We have been unable to ascertain whether the skin in the fibrotic area or a chronic wound in the diabetic has higher levels of AGEs when compared to the inside of the arm where measurements are usually taken (Corstjens et al., 2008). Therefore, we are currently in the process of elucidating why the fibrosis would predominantly appear on the back of the shoulders. One conjecture is that AGEs are not deposited in the skin evenly and they may be sequestered more within certain areas of the body. This could be due to a number of reasons, including the variation in skin thickness throughout the body (Lee and Hwang, 2002) and therefore the ECM content in those areas would also vary. Indeed, the results presented here start to explain the decreased collagen expression in the skin for chronic wounds and the increased collagen expression for fibrosis that are observed in diabetic patients.

We have reason to believe that the signaling in two pathways is affected by 3DG-collagen and MG-collagen which is mediated by the integrin receptor (Fig. 5) and the TGF-β1 receptor. We found focal adhesion kinase expression is decreased when fibroblasts are cultured on 3DG-collagen (Loughlin and Artlett, 2009) and speculated that the AKT pathway that signals through ERK1/2 is also affected and therefore we assessed ERK1/2. We found that ERK1/2 phosphorylation was decreased with 3DG-collagen (Loughlin and Artlett, 2009). AKT phosphorylation is also able to mediate the activity of Sp1, thus directly affecting COL1A1 expression (Bujor et al., 2008) and indeed we find that Sp1 activity is decreased with 3DG (data not shown). One intermediary molecule that focal adhesion kinase signals through is phosphatidylinositol 3-kinase and this protein is known to activate protein kinase C resulting in the downstream activation of TGF-β1 receptors (Yasuda et al., 2001), thereby leading to the regulation of the collagen gene through Smad7 (Zhang et al., 2004).

Skin aging is characterized by the progressive deterioration of its functional properties that are linked to alterations of the dermal connective tissue. It is apparent that older skin heals more slowly than skin from younger individuals, and aged and diabetic skin exhibits

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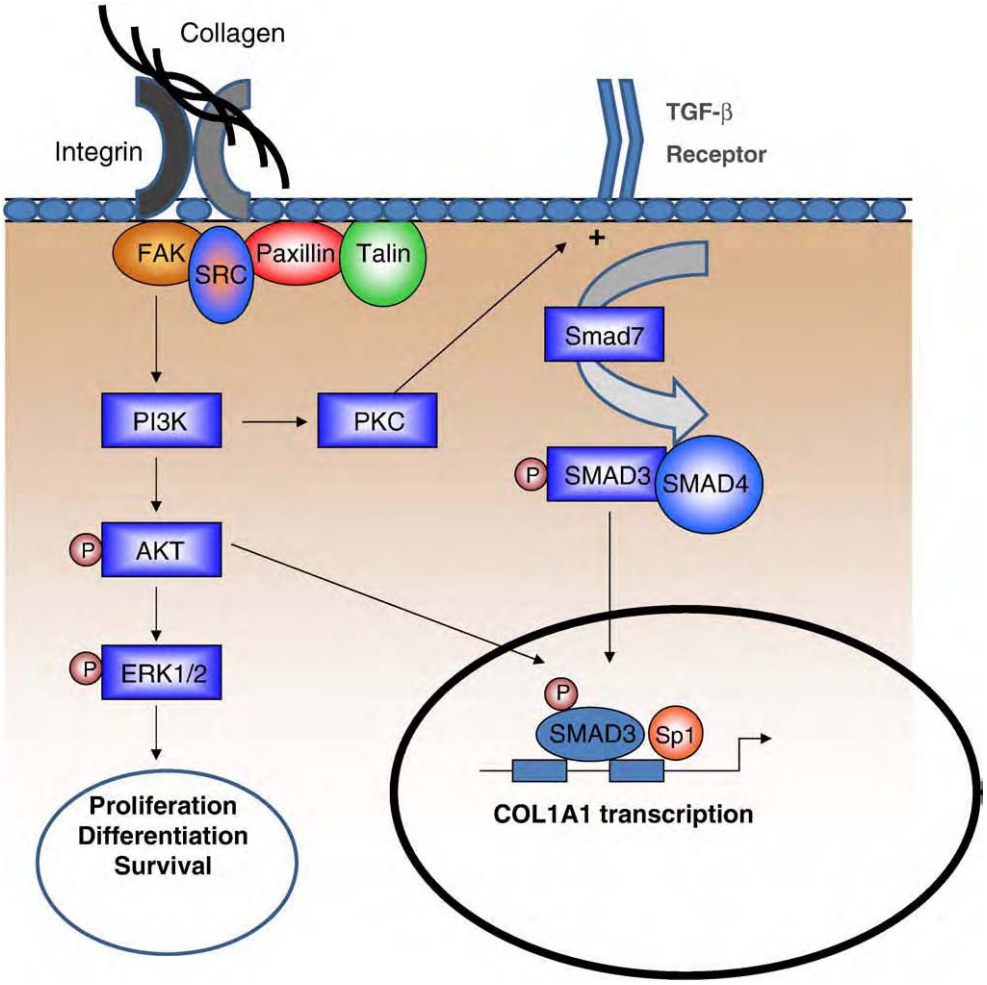

Fig. 5. Altered cellular signaling in fibroblasts mediated by 3DG or MG. We have reason to believe that the signaling in two pathways is affected by 3DG-collagen and MG-collagen which is mediated firstly through the integrin receptor and then through the TGF-β1 receptor. Focal adhesion kinase signals though phosphatidylinositol 3-kinase (PI3K), AKT (also called protein kinase B) and extracellular signal-regulated kinase 1/2 (ERK1/2). Activation of this pathway induces differentiation, proliferation, and survival. AKT phosphorylation is also able to mediate the activity of Smad3 and the binding of Sp1 to the collagen promoter, thus directly affecting COL1A1 expression. PI3K is known to activate protein kinase C (PKC) resulting in the activation of TGF-β1 receptors. If Smad7 is elevated, this inhibits the nuclear translocation of Smad3 thereby inhibiting collagen expression.

similar properties in wound healing including decreased collagen gene expression and increased apoptosis. The interaction of fibroblasts with the ECM is known to be crucial for processes as widely varied as embryonic organ development and wound healing. Tissue regeneration during superficial wound healing is impaired in the elderly, and in individuals with circulatory disorders, especially diabetics. One possible therapy for diabetics and other patients who have chronic wounds would be to inhibit 3DG, whereas to inhibit scleredema diabeticorum the inhibition of MG may be of therapeutic benefit. Further analysis of 3DG and MG is required to understand this dichotomy in collagen expression with these two AGE precursors and will yield useful clues to their role in fibrosis or chronic wounds in patients with diabetes.

4. Experimental procedures

This study was approved by the Internal Review Board of Drexel University for human studies.

4.1. In vitro cross-linking of collagen by 3DG and MG

Acid extracted type I collagen (95–97% COL1A1; 3–5% COL3A1) from human skin was purchased from Stem Cell Technologies, Vancouver BC. In vitro cross-linking of human collagen was performed at 37 °C with increasing time; 1, 4, 18, 24, and 48 h. Thirty micrograms of collagen diluted in phosphate buffered saline was incubated with either 1 mM 3DG, 1 mM MG 1 mM 3DG with 1 mM aminoguanidine (AG), 1 mM MG with 1 mM AG. COL1A1 was size fractionated on 6% SDS PAGE gels (Invitrogen, Carlsbad CA) for 3 h at 200 V. The proteins were stained with Safe Stain (Invitrogen) for 60 min and then destained overnight with tap water.

4.2. Tissue culture

A total of nine different human fibroblasts cell lines were employed in these experiments (GM05399, GM05659, GM00498, GM00731, GM0024, GM0321, GM01706, GM6112, and GM03525) and were purchased from the Coriell Institute for Medical Research (Camden NJ). All lines were established from normal skin biopsies and cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin and glutamine. Morpholinofructose (MF), MG, and AG were obtained from Sigma-Aldrich, St Louis MO. 3DG was obtained from Toronto Research Chemicals, North York Ontario.

4.3. Preparation of collagen lattices

Human collagen solution was diluted in PBS to 0.067 mg/ml according to Kessler (Kessler et al., 2001). The culture dishes were flooded with

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collagen and incubated for 2 h at 37 °C. Dishes were gently washed three times with sterile PBS and used immediately or the collagen further modified with 1 mM 3DG, or 0.1 nM MG overnight. Unincorporated 3DG or MG was removed from the plates by gently washing the matrix three times with 5 ml PBS prior to plating with fibroblasts.

4.4. 3DG measurements

Fibroblasts were seeded into 70 mm culture dishes in complete DMEM and allowed to adhere overnight prior to the addition of MF (10 mM) for 24 h. After 24 h, an aliquot of the media was frozen at −80 °C for analysis for 3DG. The fibroblast cells were then washed 3 times with PBS and detached from the dish with trypsin and the cells pelleted by centrifugation (12,000*g*) and stored at −80 °C until assayed for 3DG. 3DG in the media and the cell pellet was extracted and measured according to Lal et al. (1997) with modifications. 3DG in the media was measured by incubating 50 µl of media, 20 µl of 10 µM [U-C13]-3DG (as internal standard) and 1 ml of 0.1% 2,3-diaminonaphthalene in 50 mM phosphate buffer, pH 7.4 at 20 °C for 18 h. The mixture was extracted with 2 ml of ethyl acetate and the ethyl acetate extract evaporated. The resulting residue was dissolved in 200 µl of ethyl acetate and transferred to a 250 µl limited volume insert and evaporated. The residue was treated with 100 µl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide and heated for 30 min at 50 °C. The samples were analyzed using an Agilent Technologies 6850 Series II Network GC system equipped with a 6850 Auto Sampler and a 5975B insert MSD. One microliter of sample was injected and the GC separation was performed on a fused silica capillary column from Agilent (DB-1701, 30 m × 0.25 mm i.d.). The GC/MS temperature program was injector port 250 °C and initial column temperature 100 °C for 1 min, then ramp to 200 °C at 10 °C/min and hold for 1 min then ramp to 280 °C at 20 °C/min and hold for 14 min. The transfer line was maintained at 250 °C. Quantification of 3DG was performed by selected ion monitoring using the *m/z* fragment of 295 for media and cellular 3DG and *m/z* fragment of 299 for the [U-13C]-3DG internal standard. For cell pellets 100 mg of pellet and 20 µl of 10 µM [U-C13]-3DG was homogenized in 600 µl of ice cold 5% $HClO_4$. The suspension was centrifuged and the supernate adjusted to ~pH 7 with 1 N KOH. The precipitated $KClO_4$ was removed by centrifugation and the supernate added to 1 ml of 0.1% 2,3-diaminonapthalene in 50 mM phosphate buffer. This was then processed as above.

4.5. Hydroxyproline assays

The media from fibroblasts cultured with MF (10 mM) was assayed for hydroxyproline according to the established method of Woessner (1961). One milliliter of media was hydrolyzed with an equal volume of HCl overnight at 110 °C, dried, resuspended in 1.0 ml chloramine-T, 1.0 ml perchloric acid, and 20% wt/vol p-dimethyl-amino-benzealdehyde and heated at 60 °C for 20 min. Once the tubes were cool the sample was read in a spectrophotometer at 557 nm and the concentration of hydroxyproline was determined against a standard curve.

4.6. SYBR green quantitative RT-PCR

Total RNA from confluent fibroblasts was extracted using the RNeasy Mini kit (Qiagen, Valencia CA) according to the manufacturer's protocol. To verify the expression of COL1A1, COL3A1, TGF-β, and β1-integrin; 2.0 µg of total RNA was reverse-transcribed using Super-script-III reverse transcriptase (Invitrogen), according to manufacturer's protocol. Transcripts were quantified using SYBR Green PCR amplification (Perkin Elmer, Waltham MA). The following primers were employed to detected transcripts of interest: COL1A1-forward: 5′-CCAGAAGAACTGGTACATCAGCA-3′and COL1A1-reverse: 5′-CGCCATACTCGAACTGGAAT-3′; COL3A1-forward 5′-TTTGGCACAACAGGAAGCTG-3′ and COL3A1-reverse 5′-GGACTGACCAAGATGGGAACAT-3′; TGF-β-forward 5′-CGAGCCTGAGGCCGACTAC-3′ and TGF-β-reverse 5′-AGATTTCGTTGTGGGTTTCCA-3′; β1-integrin-forward 5′-CAAAGGAACAGCAGAGAAGC-3′ and β1-integrin-reverse 5′-ATTGAGTAAGACAGGTCCATAAGG-3′. All mRNA transcripts were normalized to β-actin expression using the following primers: β-actin-forward 5′-TTGCCGACAGGATGCAGAA-3′ and β-actin-reverse 5′-GCCGATCCACACGGAGTACTT-3′.

4.7. Protein analysis and Western blotting

Collagen matrices in 70 mm dishes were cross-linked with increasing amounts of 3DG (1 mM, 2 mM, or 4 mM) or with MG (0.01 nM, 0.1 nM, or 1 nM) overnight and the matrices washed as described above. Fibroblasts were seeded onto the matrices so that the cells were confluent and cultured for 48 h before lysing in Tissue Extraction Reagent II (Invitrogen) supplemented with protease inhibitors (Sigma-Aldrich). Thirty-micrograms of total protein was size fractionated on a 10% SDS polyacrylamide gel (Invitrogen) and the proteins transferred to a PVDF membrane (Invitrogen). The membrane was blocked in 5% skim milk and probed with mouse-anti-human COL1A1 or COL3A1 (Santa Cruz) diluted 1:500 overnight at room temp with rocking. The membrane was washed three times with TBS-Tween to remove any unbound proteins and incubated with a secondary antibody, rabbit-anti-mouse-HRP (1:3000) (Jackson Immunoresearch, West Grove PA). The HRP signal was developed with SuperSignal Chemiluminescent Substrate (Pierce). The intensity of the bands was measured by ImageJ (http://rsbweb.nih.gov/ij/) and protein expression was assayed according to β-actin protein. TGF-β1 and β1-integrin were also analyzed by Western blotting and normalized to β-actin protein. Native collagen was modified with either 1 mM 3DG or 0.1 nM MG as described and fibroblasts cultured on the modified collagen matrices for 24 h, then protein harvested and subjected to Western blotting as described above. TGF-β1, Smad7, and β1-integrin antibodies were purchased from Santa Cruz and both were diluted to 1:500 in PBS prior to use.

ELISA for active TGF-β1 was performed on the culture supernatants using the TGFβ1 Emax ImmunoAssay System (Promega, Madison WI) according to the manufacturer's protocol.

4.8. Statistical analysis

The resulting data were subjected to 2-tailed Paired *T* Test for statistical significance. A P value of <0.05 was considered significant.

Conflicts of interest

Drs. Kappler, Schwartz, Su, and Tobia are employees and shareholders of Dynamis Therapeutics, Inc. Dr. Artlett is a shareholder of Dynamis Therapeutics, Inc. Drexel University was the recipient of a research grant from Dynamis Therapeutics, Inc.

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Precursor of Advanced Glycation End Products Mediates ER-Stress-Induced Caspase-3 Activation of Human Dermal Fibroblasts through NAD(P)H Oxidase 4

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Abstract

Background: The precursor for advanced glycation end products, 3-deoxyglucosone (3DG) is highly upregulated in skin explants of diabetic cutaneous wounds, and has been shown to negatively impact dermal fibroblasts, which are crucial in wound remodeling. 3DG induces apoptosis however; the mechanisms involved in the apoptotic action of 3DG in the pathogenesis of diabetic chronic wounds are poorly understood. Therefore, we sought to delineate novel mechanisms involved with the 3DG-collagen induced apoptosis.

Methodology/Principal Findings: Using human dermal fibroblasts, we demonstrated that 3DG-modified collagen induces oxidative stress and caspase-3 activation. Oxidative stress was found to be dependent on the upregulation of NAD(P)H oxidase 4 (Nox4), a reactive oxygen species (ROS) Nox homologue, triggering endoplasmic reticulum (ER) stress, as assessed by the ER stress-induced apoptosis marker Growth Arrest and DNA Damage-inducible gene 153 (GADD153). We demonstrated that 3DG-collagen activated GADD153 via phosphorylation of p38 mitogen activated protein kinase (MAPK), and this was dependent on upstream ROS. Inhibition of ROS and/or p38 MAPK abrogated 3DG-collagen induced caspase-3 activation. Our investigations also demonstrated that 3DG-collagen-induced caspase-3 activation did not signal through the canonical receptor for advanced glycation end products (RAGE) but through integrin $\alpha1\beta1$. To further verify the role of integrins, neutralization of integrins $\alpha1\beta1$ prevented 3DG-collagen-induced upregulation of ROS, GADD153, and caspase-3 activation; suggesting that 3DG-collagen signaling to the fibroblast is dependent on integrins $\alpha1\beta1$.

Conclusions/Significance: Taken together, these findings demonstrate for the first time that a RAGE independent mechanism is involved in 3DG-collagen-induced apoptosis. Moreover, the ER stress pathway through activation of Nox4 by integrins $\alpha1\beta1$ plays a key role in 3DG-collagen-induced caspase-3 activation, which may play an important role in the pathogenesis of diabetic wounds.

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Introduction

Wound healing is impaired in patients with diabetes as approximately 5–8% of the patients develop chronic foot ulcers [1–3]. Patients suffering from chronic diabetic ulcers have increased levels of apoptosis within their infected tissues and this may interfere with their capacity to efficiently heal wounds [1,3–7]. The high degree of cell death seen in fibroblasts explanted from the skin of diabetic patients may be partly due to the formation of advanced glycation end products (AGEs) [8]. AGEs result from a non-enzymatic reaction between glucose and protein, which can form irreversible cross-links on long-lived proteins such as collagen [6,9–11]. One precursor for AGEs is the highly reactive α-dicarbonyl 3-deoxyglucosone (3DG). 3DG has been shown to play a role in the modification and cross-linking of collagen [9,10,12,13]. One mechanism by which 3DG-modifed collagen could affect wound healing is through increased apoptosis.

The role of AGE induction of apoptosis has been investigated extensively, however many of these studies were performed on various cell types including neuronal and endothelial cells [14–20] using soluble AGEs, resulting in differing apoptotic responses [8,20–22]. Soluble AGEs induce apoptosis in endothelial and neuronal cells through engagement of its receptor, receptor for advanced glycation end products (RAGE) [15–20]. This ligand-receptor engagement promotes the upregulation of reactive oxygen species (ROS) resulting in apoptosis [19,23–25]. Moreover, reports utilizing AGEs cross-linked to collagen agree with the current view that AGEs induce apoptosis; however, the mechanisms by which AGE-modifed collagen does so are conflicting and not well understood [8]. More importantly, none of the studies to date have investigated 3DG or its signaling events that induce apoptosis in dermal fibroblasts.

One possibility for the conflicting evidence is due to the varying types of precursors responsible for the development of AGEs [9,10,26]. We previously demonstrated that 3DG-collagen signals to

the fibroblast in an anti-fibrotic way, causing decreased fibroblast migration, proliferation, and extracellular matrix (ECM) production [27–29]. In contrast, methylglyoxal (MG), a well studied AGE precursor, has been implicated in pro-fibrotic conditions such as atherosclerosis, and modification of collagen by MG has been shown to increase fibroblast proliferation and ECM production [10,29–31]. Because the varying pathology of the AGEs in diabetes could rely on the dicarbonyl that produces them, it is important to understand the role of apoptosis in the context of independent AGE precursors. Therefore, we investigated the mechanism through which 3DG-collagen induces apoptosis in human dermal fibroblasts.

Previous work in our laboratory has revealed a role for the endoplasmic reticulum (ER) stress signaling pathway in promoting apoptosis of dermal fibroblasts cultured on 3DG-collagen [28]. ER stress can be induced by various stressors on the cell including oxidative stress [32–37]. ROS can be formed through activation of the NAD(P)H oxidase system [38–40] and imbalanced activation of this oxidase system can lead to oxidative stress [39,41]. ROS have been shown to disrupt ER homeostasis resulting in the accumulation of misfolded proteins and induction of ER stress [35–37,42]. ROS induces the transcription factor Growth Arrest and DNA Damage inducible gene 153 (GADD153), also known as C/EBP homologous protein, through phosphorylation of the stress activated kinase p38 MAPK [35,37,43–45], where its activation is considered to be a classic marker of ER stress-induced apoptosis [33,34,43,46–48]. Induction of GADD153 causes the cell to undergo apoptosis through the upregulation of caspase-3 [37,43]. Upon investigation, GADD153 was found to be highly upregulated in fibroblasts cultured on 3DG-collagen, while its expression was downregulated in fibroblasts treated with the 3DG inhibitor meglumine [28]. To date, there are no reports suggesting a possible mechanism by which 3DG-collagen induces the apoptosis signaling cascade in dermal fibroblasts. Therefore, this study was undertaken to further delineate the 3DG-collagen signaling mechanism with emphasis on the ER stress pathway.

Results

ER stress mediates 3DG-collagen-induced caspase-3 activation in human dermal fibroblasts

To test whether 3DG-collagen induces the apoptotic signaling cascade in human dermal fibroblasts and determine if it was dependent on ER stress, fibroblasts were cultured on native collagen or 3DG-collagen coated dishes with or without aminoguanidine (AG), or treated with or without meglumine for 24 h. Apoptosis was measured by the expression of active caspase-3, an early marker of apoptosis. Fibroblasts cultured on 3DG-collagen induced a 150%±4.5% increase in active caspase-3 expression compared to fibroblasts grown on native collagen (Figure 1, $p<0.0002$). When fibroblasts were treated with AG, which is known to inactivate 3DG's ability to cross-link collagen, the activity of caspase-3 was reduced to levels observed in fibroblasts grown on native collagen. Additionally, meglumine, an inhibitor of 3DG, prevented the 3DG-collagen-induced increase in caspase-3 activity (Figure 1).

ER stress is known to induce apoptosis of the cell [32–35,37,43,46,48]. Therefore, fibroblasts were pretreated with the ER stress inhibitor salubrinal and then cultured on native collagen or 3DG-collagen for 24 h. Treatment with salubrinal reduced the level of active caspase-3 within fibroblasts cultured on 3DG-collagen to that of fibroblasts cultured on native collagen (Figure 1, $p<0.0002$). These results suggest that 3DG-collagen can induce the activation of the apoptotic signaling cascade through ER stress.

ER stress-induced apoptosis marker GADD153 is upregulated in fibroblasts cultured on 3DG-collagen

Accumulation of misfolded proteins within the ER can lead to stress and induction of GADD153, a transcription factor involved in apoptosis [32–35,37,43,46–48]. Previously, we demonstrated the upregulation of GADD153 transcript levels and activation of GADD153 in fibroblasts by 3DG-collagen [28]. Moreover, meglumine was found to inhibit 3DG-collagen-induced GADD153 expression [28]. To further confirm that 3DG-collagen is inducing ER stress, fibroblasts were pretreated with salubrinal, an inhibitor of ER stress, and cultured on 3DG-collagen for 24 h. The fibroblasts were then stained for GADD153 and inspected for GADD153 localization within the nucleus, which is indicative of activated GADD153 [27] and mean fluorescent intensity (MFI) of the nuclei was measured. In the presence of salubrinal, fibroblasts cultured on 3DG-collagen reduced the expression of GADD153 to that observed in fibroblasts cultured on native collagen (11.2 MFI±2.0 with salubrinal compared to 30.5 MFI±2.1 on 3DG-collagen), further confirming that 3DG-collagen is inducing ER stress in dermal fibroblasts (Figure 2A, $p<0.007$). To verify the immunofluorescence results, a Western blot was performed. Confirming the

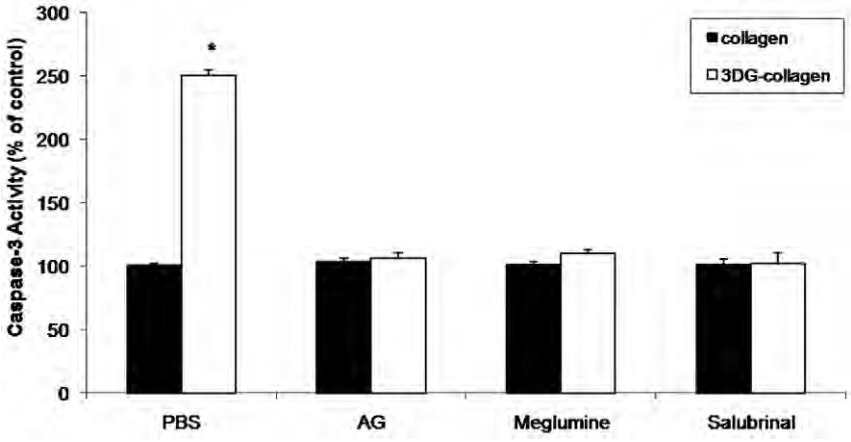

Figure 1. Induction of caspase-3 activity by 3DG-collagen is dependent on ER stress. Fibroblasts were cultured on native collagen or 3DG-collagen and treated with or without 5 mM AG, 40 mM meglumine, or 40 μM salubrinal for 24 h. 100 μg of whole cell lysate was assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against collagen treated with PBS. Data are mean±SD (n = 3), *$P<0.0002$.
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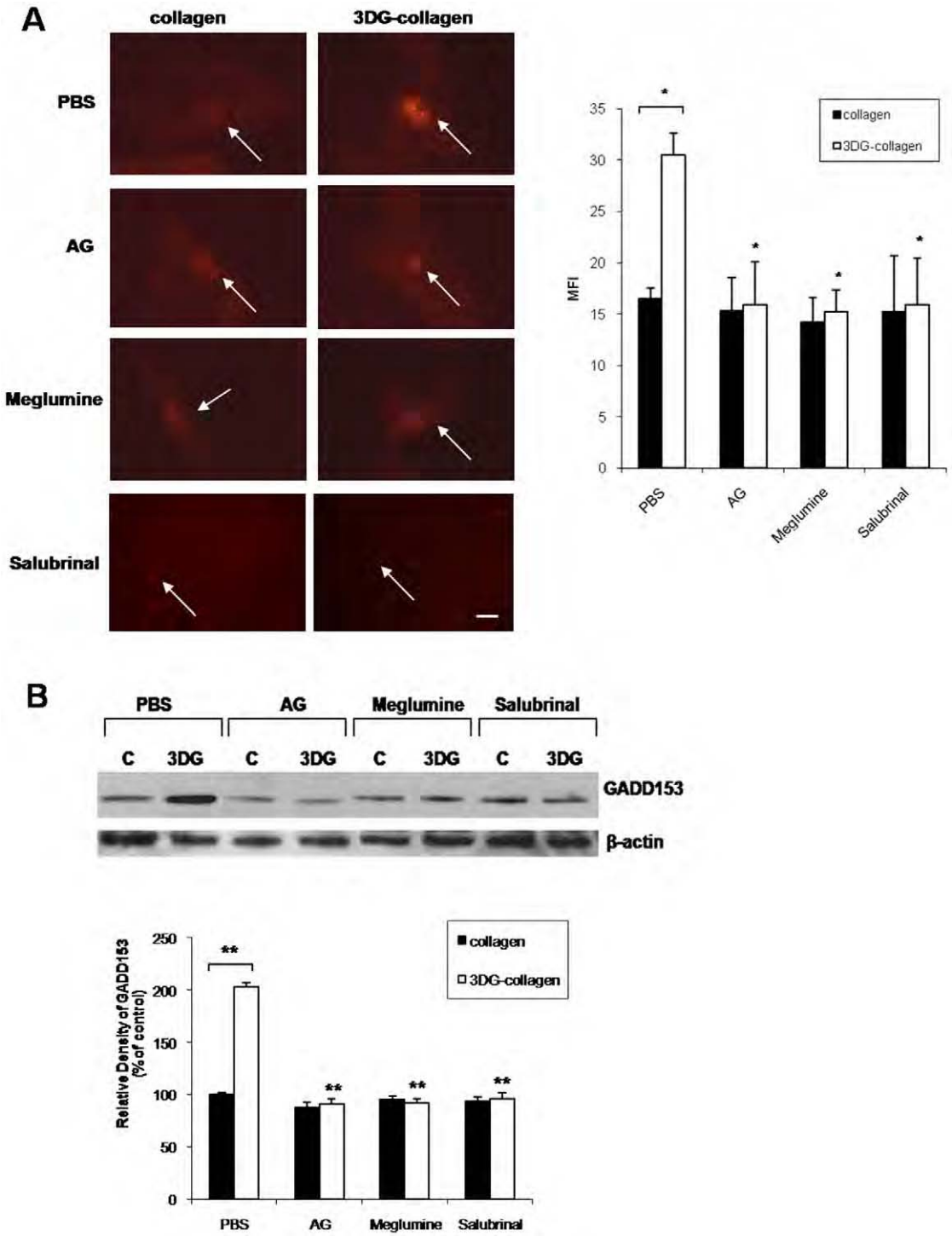

Figure 2. Effect of ER stress inhibitor salubrinal on 3DG-collagen-induced GADD153 expression. A, Fibroblasts were cultured in chamber slides coated with native collagen or 1 mM 3DG-collagen with or without 5 mM AG or 40 mM meglumine for 24 h. Also, fibroblasts were pretreated for 1 h with or without 40 μM salubrinal and then cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts were stained and analyzed for expression of GADD153 in the nucleus by immunofluorescence analysis using Cy3-conjugated secondary antibody. Mean fluorescence intensity (MFI) of GADD153 in the nucleus was measured using ImageJ from ten representative fibroblasts. Images were taken at 40× magnification on an epi-fluorescent microscope. Arrows indicate nuclei containing GADD153. The bars represent the MFI values from each experimental condition. Scale bar represents 10 μm. **B**, Fibroblasts were treated as in **A** followed by Western blot for GADD153 expression. β-actin was used as a loading control. The bars represent the densitometric value for each experimental condition. All comparisons are made against 3DG-collagen treated with PBS unless otherwise indicated. Data are mean±SD (n = 3), **P<0.0005, *P<0.007.
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immunofluorescence observations, Western blot analysis demonstrated an increase to 203%±4.1% in GADD153 expression in fibroblasts cultured on 3DG-collagen compared to fibroblasts cultured on native collagen ($p<0.0005$). AG and meglumine reduced the level of GADD153 expression in fibroblasts cultured on 3DG-collagen to 91%±5.2% and 92%±4.2% ($p<0.0005$), respectively and salubrinal prevented the 3DG-collagen-induced increase of GADD153 ($p<0.0005$) (Figure 2B). These findings suggest that 3DG-collagen is inducing apoptosis through the ER stress-signaling pathway, which is dependent on GADD153 activation.

3DG-collagen stimulates ROS in dermal fibroblasts

ROS are known to cause oxidative stress and have been linked to the activation of GADD153-induced apoptosis in cells [35,41,43,46,49,50]. Therefore, we determined if ROS were produced during the culturing of fibroblasts on 3DG-collagen. Fibroblasts cultured on 3DG-collagen produced 376 nM±3.4 of intracellular ROS at 24 h in comparison to the 38.7 nM±2.2 of ROS produced by fibroblasts grown on native collagen (Figure 3, $p<0.001$). This increase was comparable to that observed with hydrogen peroxide (H_2O_2), a free radical involved in ER stress, which produced 458 nM±3.2 (Figure 3, $p<0.001$). Moreover, AG abrogated the rise in ROS only in fibroblasts cultured on 3DG-collagen, and not in cells treated with H_2O_2 suggesting that 3DG-collagen is specifically producing ROS (Figure 3; 94.3 nM±4.0 for 3DG-collagen/AG; 440.6 nM±4.0 for H_2O_2/AG, $p<0.001$). Meglumine inhibited the production of ROS in cells cultured on 3DG-collagen and partially inhibited ROS in cells treated with H_2O_2 suggesting that meglumine may prevent ROS induction by 3DG-collagen and/or may be a scavenger of free radicals (92.8 nM±4.0 for 3DG-collagen and meglumine; and 210.2 nM±4.4 for H_2O_2 and meglumine, $p<0.001$). In addition, the induction of ROS by fibroblasts cultured on 3DG-collagen, or treated with H_2O_2 could be blocked by pretreating fibroblasts with the antioxidant ascorbic acid (Figure 3; 78.2 nM±3.6 for 3DG-collagen/ascorbic acid; and 55.6 nM±4.8 for H_2O_2/ascorbic acid, $p<0.001$). Taken together, these results suggest that ROS could be produced in the fibroblast in response to the modification of collagen by 3DG.

NAD(P)H oxidase 4 is responsible for the 3DG-collagen-dependent production of ROS

The NAD(P)H oxidase (Nox) controls the production of ROS through integrin activation, and cytokine and growth factor stimulation [38,40,51,52]. Overexpression of key oxidases such as the non-phagocytic Nox4 has been associated with increased ROS and apoptosis [38,39,53]. Nox4 has been shown to be highly expressed in fibroblasts compared to other Nox homologues [38,39]. Therefore, we determined if 3DG-collagen-induced ROS were mediated by the overexpression of Nox4. Quantitative real-time PCR revealed that Nox4 mRNA expression increased to 880%±200.0% in fibroblasts cultured on 3DG-collagen for 24 h compared to fibroblasts cultured on native collagen (Figure 4A, $p<0.02$). Moreover, to ensure that Nox4 was the only Nox isoform being overexpressed by 3DG-collagen, quantitative real-time PCR was performed to determine the mRNA transcript levels of the other Nox isoforms, Nox1 and Nox2. Detection of Nox1 and Nox2 mRNA transcripts was not apparent suggesting that dermal fibroblasts overexpress specifically Nox4 (Figure 4A). To show specificity of 3DG, AG and meglumine reduced the transcript levels of Nox4 in fibroblasts cultured on 3DG-collagen to that observed in fibroblasts cultured on native collagen. Additionally, Nox4 protein levels were found to be increased in fibroblasts cultured on 3DG-collagen compared to fibroblasts cultured on native collagen (240%±8.6% in 3DG-collagen treated vs. 100%±3.3% in native collagen treated cells, Figure 4B, $p<0.001$). This upregulation was also abrogated by the 3DG inhibitors AG and meglumine. Immunofluorescence demonstrated increased Nox4 localization at the plasma membrane in fibroblasts cultured on 3DG-collagen and that this increase was abrogated by AG and meglumine, suggesting that Nox4 may be activated on the cell surface (Figure 4C).

We further investigated the role of Nox4 in the upregulation of ROS in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with apocynin, a broad class Nox inhibitor, cultured on either native collagen, 3DG-collagen, or treated with H_2O_2 for 24 h, and intracellular ROS was quantified. Apocynin reduced ROS in fibroblasts cultured on 3DG-collagen to that observed in fibroblasts cultured on native collagen (Figure 4D, $p<0.001$). This further confirms that 3DG-collagen is inducing ROS through

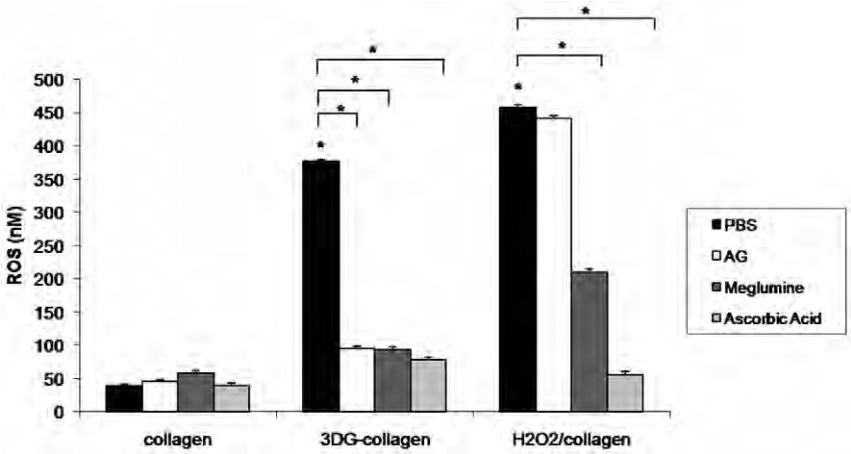

Figure 3. 3DG-collagen stimulates intracellular ROS in fibroblasts. Fibroblasts were cultured in 96-well plate coated with either native collagen or 1 mM 3DG-collagen or treated with or without 5 mM AG, 40 mM meglumine, or 100 μg/mL ascorbic acid for 24 h. Treatment of fibroblasts cultured on native collagen with 50 μM H_2O_2 was used as a positive control. Fibroblasts were loaded with DCFH-DA for 30 min and ROS production was measured by absorbance of fluorescent DCF at a wavelength of 480 nm/530 nm. Comparisons are made to collagen treated with PBS unless otherwise indicated. Data are mean±SD (n = 3), *$P<0.001$.
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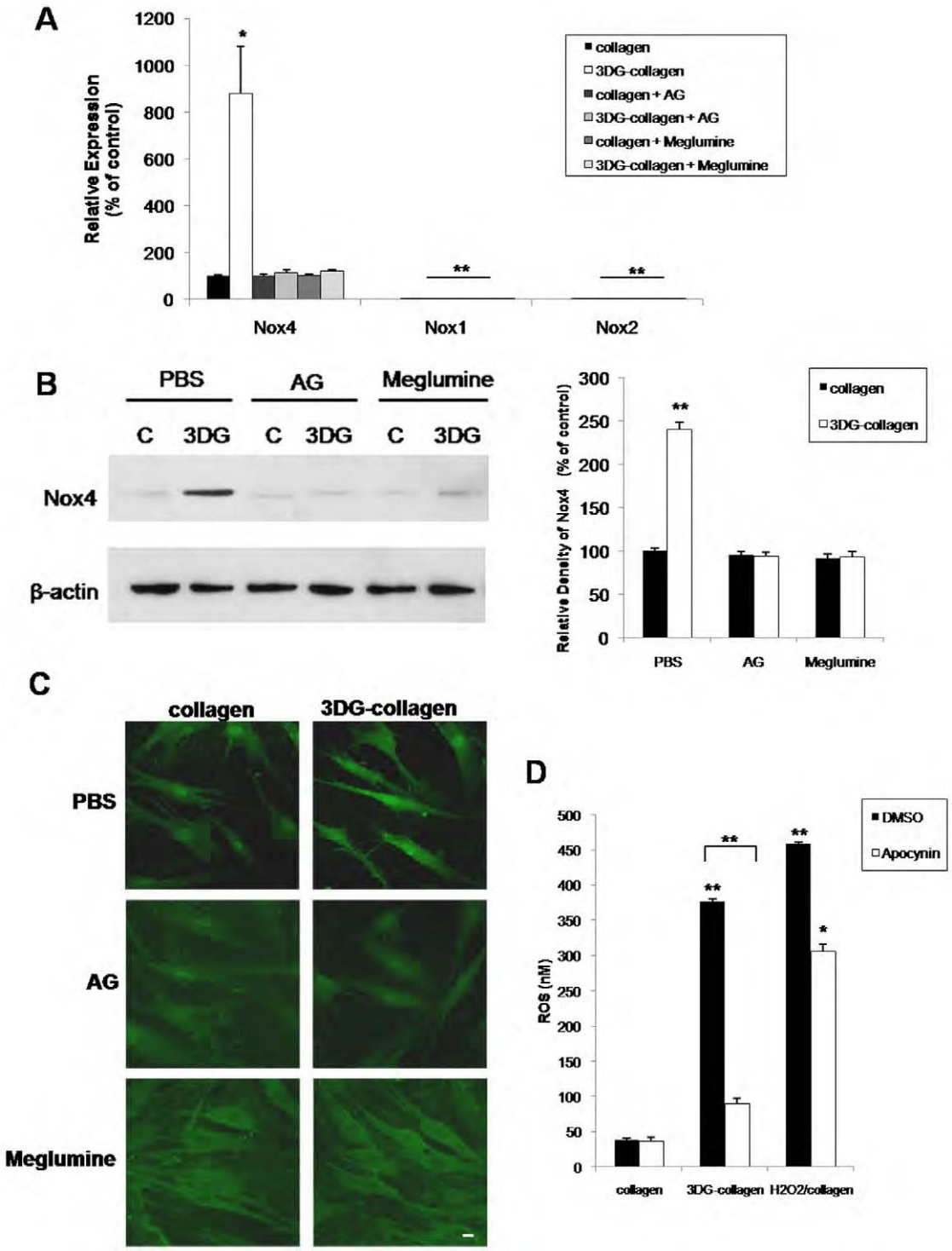

Figure 4. 3DG-collagen increases expression of Nox4 in the dermal fibroblast. Fibroblasts were cultured on either native collagen or 1 mM 3DG-collagen and treated with or without 5 mM AG or 40 mM meglumine for 24 h. **A**, Nox4, Nox1, and Nox2 mRNA expression levels were quantified by real-time PCR. All transcripts were normalized to β-actin. **B**, Expression levels of Nox4 were analyzed by Western blot and β-actin served as a loading control. Results were quantified by densitometric scanning of the Western blot and normalized for β-actin. **C**, Localization of Nox4 in fibroblasts treated the same as in **A** and **B** was analyzed by immunofluorescence with the anti-Nox4 polyclonal antibody and Cy2-conjugated secondary antibody. Images were taken at 40× magnification on an epi-fluorescence microscope. Scale bar represents 10 μm. **D**, Inhibition of Nox4 reduces the level of intracellular ROS. Fibroblasts were pretreated for 1 h with either vehicle, DMSO or NOX inhibitor, apocynin (1 mM) and cultured on native collagen, 1 mM 3DG-collagen, or cultured on native collagen and treated with 50 μM H_2O_2 for 24 h. Fibroblasts were then incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. Comparisons are made against collagen treated with DMSO and/or PBS unless otherwise indicated. Data are mean±SD (n = 3), **P<0.001, *P<0.02.
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activation of Nox4. Furthermore, apocynin was found to only partially inhibit the level of ROS in fibroblasts cultured on native collagen with H_2O_2 suggesting that apocynin is inhibiting the ROS induced by the Nox4 complex rather than affecting the induction of ROS by exogenous H_2O_2 (Figure 4D, $p<0.02$).

3DG-collagen-induced phosphorylation of p38 MAPK is dependent on upstream ROS

During times of ER stress, ROS have been shown to activate the stress kinase p38 MAPK [35,37,44]. To determine if 3DG-collagen-induced ROS are responsible for increased phosphorylation of p38 MAPK, fibroblasts were pretreated with the antioxidant ascorbic acid and the Nox inhibitor apocynin, and cultured on either native collagen, 3DG-collagen, or native collagen and treated with H_2O_2 and protein levels were measured by Western blotting. Fibroblasts cultured on 3DG-collagen increased the phosphorylation of p38 MAPK to 175%±4.1%. As a positive control for ROS-induced p38 MAPK activation, fibroblasts cultured on native collagen and treated with H_2O_2 increased the phosphorylated p38 MAPK to 181%±3.3%. Pretreatment with ascorbic acid reduced the phosphorylation of p38 MAPK in fibroblasts cultured on 3DG-collagen or treated with H_2O_2 to that seen in fibroblasts cultured on native collagen (Figure 5, $p<0.0001$). Additionally pretreatment with apocynin reduced the level of phosphorylated p38 MAPK in fibroblasts cultured on 3DG-collagen, but not in fibroblasts treated with H_2O_2 (Figure 5, $p<0.0001$). These results suggest that 3DG-collagen-induced p38 MAPK is dependent on upstream production of ROS by Nox4.

3DG-collagen-induced GADD153 expression is dependent on upstream ROS and p38 MAPK activation

3DG-collagen-induced ROS can lead to phosphorylation of p38 MAPK, which is essential for the activation of GADD153; therefore, the functional role of ROS and p38 MAPK in GADD153 induction was assessed [35,37,43,44]. To determine whether GADD153 induction by 3DG-collagen was a result of free radical-mediated effects, fibroblasts were pretreated with ascorbic acid or apocynin and then cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts cultured on native collagen and treated with H_2O_2 were used as a positive control for ROS-induced GADD153 activation. The trafficking of GADD153 from the cytosol to the nucleus was found to be downregulated to 14.6 MFI±2.1 and 16.3 MFI±0.98 in response to ascorbic acid in fibroblasts cultured on 3DG-collagen or native collagen treated with H_2O_2, respectively (Figure 6A, $p<0.007$). The expression of GADD153 in the nucleus of fibroblasts pretreated with apocynin and cultured on 3DG-collagen was also reduced to 13.9 MFI±1.2 (Figure 6A, $p<0.007$). Western blot was performed to verify the expression of GADD153. GADD153 expression was decreased in response to ascorbic acid and apocynin in fibroblasts cultured on 3DG-collagen, while only ascorbic acid reduced the level of GADD153 in fibroblasts cultured on native collagen treated with H_2O_2 (Figure 6B; 81%±2.4% 3DG-collagen treated with ascorbic

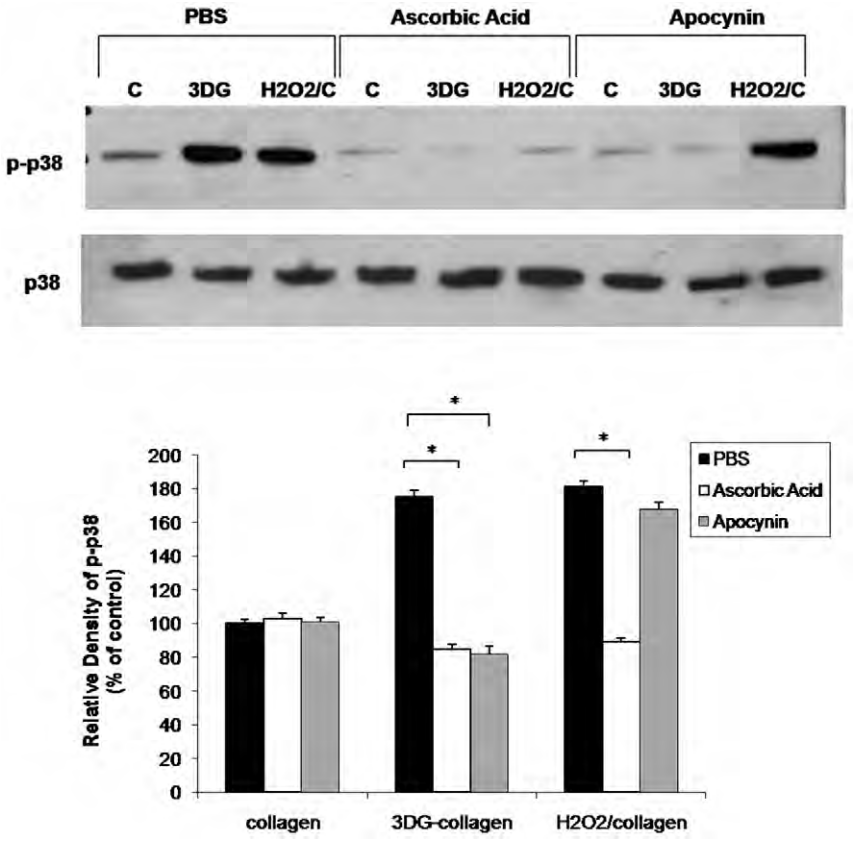

Figure 5. Phosphorylation of p38 MAPK is dependent on 3DG-collagen-induced ROS. Fibroblasts were pretreated for 1 h with 100 μg/mL of ascorbic acid, 1 mM apocynin, or DMSO and cultured on either native collagen, 1 mM 3DG-collagen, or treated with 50 μM H_2O_2 for 24 h. Whole cell lysates were extracted and Western blot analysis of p-p38 MAPK was performed. Total p38 MAPK was used as a loading control. The bars correspond to the densitometric value of p-p38 MAP kinase after normalization for total p38 MAP kinase. Data are mean±SD (n = 3), *P<0.0001.
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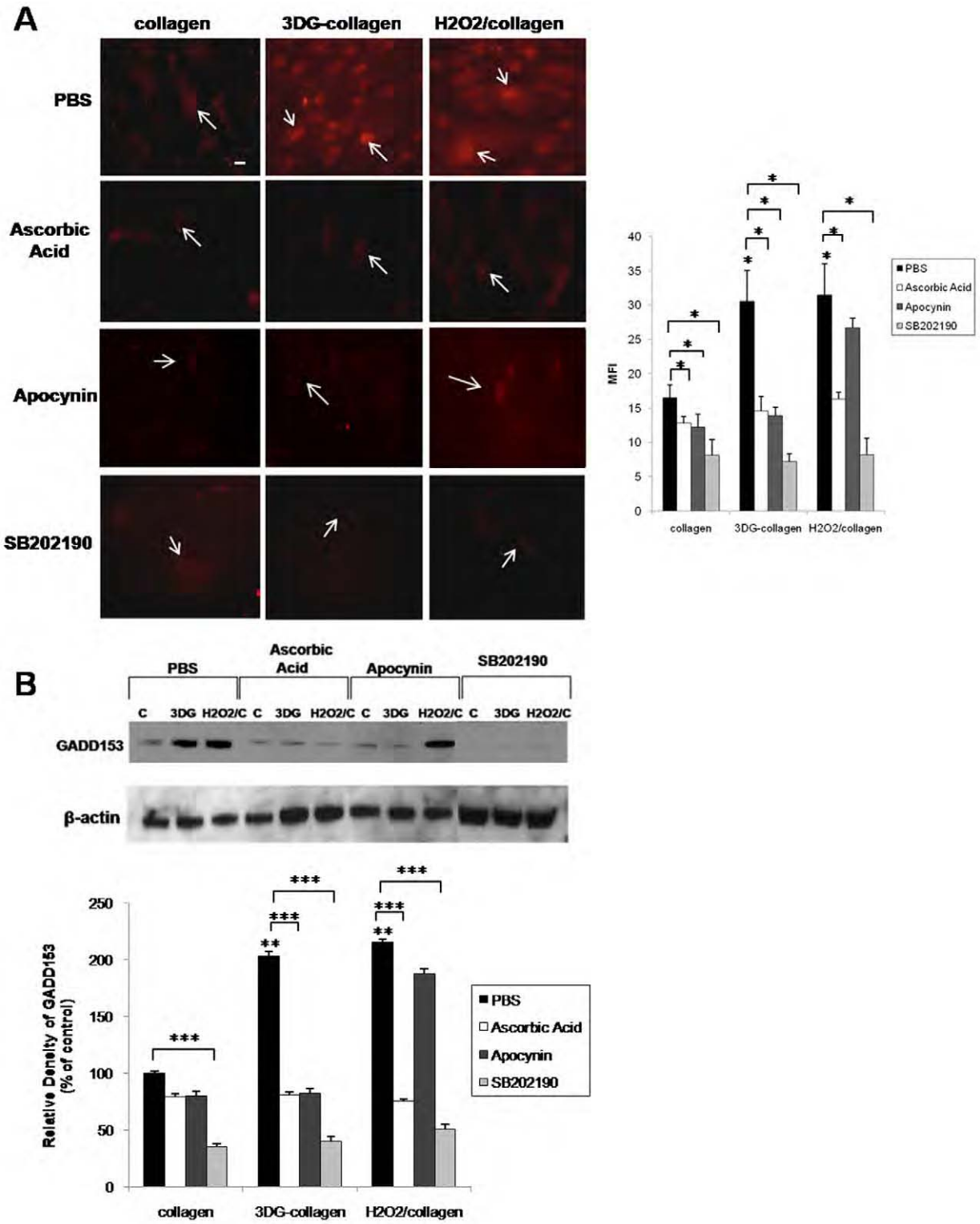

Figure 6. Inhibition of ROS, and p38 MAPK abrogates GADD153 expression in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with 100 μg/mL of ascorbic acid, 1 mM apocynin, or 10 μM SB202190 for 1 h, and cultured on native collagen, 1 mM 3DG-collagen or treated with 50 μM H_2O_2 for 24 h. **A**, Fibroblasts were stained and analyzed for the expression of GADD153 in the nucleus by immunofluorescence using a Cy3-conjugated secondary antibody. Representative images were taken at 40× magnification on an epi-fluorescence microscope, and the MFI of ten nuclei was analyzed by Image J. Bars correspond to the MFI of treated fibroblasts. Arrows indicate nuclei containing GADD153. Scale bar represents 10 μm. **B**, Western blot for the expression of GADD153 in whole cell lysates. β-actin was used as a loading control. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean±SD (n = 3), ***P<0.0001, **P<0.0005, *P<0.007.
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acid and 82%±4.6% treated with apocynin, and 75%±2.2% H_2O_2 treated with ascorbic acid and 188%±3.6% treated with apocynin, $p<0.0001$). These results suggest that the generation of ROS by Nox4 lies upstream of GADD153.

Next, the role of p38 MAPK in GADD153 activation was assessed in fibroblasts cultured on native collagen or 3DG-collagen. Fibroblasts were pretreated with the p38 MAPK inhibitor SB202190 and cultured on native collagen, 3DG-collagen, or native collagen and treated with H_2O_2 for 24 h. Inhibition of p38 MAPK by SB202190 reduced the localization of GADD153 in the nucleus to 7.23MFI±1.13% in fibroblasts cultured on 3DG-collagen (Figure 6A, $p<0.007$), and reduced the expression of GADD153 to 40%±4.0% (Figure 6A, $p<0.0001$). In addition, inhibition of p38 MAPK reduced the level of GADD153 expression in fibroblasts grown on native collagen treated with H_2O_2 to 51%±3.6% (Figure 6B, $p<0.0001$), and its nuclear localization to 8.21MFI±2.4 (Figure 6A, $p<0.007$). These results suggest that the induction of p38 MAPK by upstream ROS is responsible for the activation of GADD153 by 3DG-collagen.

3DG-collagen-induced caspase-3 activation is dependent on upstream ROS and p38 MAPK activation

A caspase-3 assay was performed to determine if ROS and p38 MAPK are responsible for the increased caspase-3 activation observed in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with ascorbic acid, the Nox inhibitor apocynin, or the p38 MAPK inhibitor SB202190; and cultured on native collagen or 3DG-collagen for 24 h, or cultured on native collagen and treated with H_2O_2 as a positive control. Fibroblasts treated with ascorbic acid, apocynin, or SB202190 and cultured on 3DG-collagen reduced the activation of caspase-3 to 116%±4.7%, 115%±4.5%, and 105%±2.5% respectively. This expression was comparable to the level of caspase-3 cleavage observed in fibroblasts cultured on native collagen and treated with H_2O_2 in the presence of ascorbic acid (112%±7.2%) or SB202190 (104.2%±6.6%), and fibroblasts cultured on native collagen (100%±1.4%; Figure 7, $p<0.0002$). This data suggests that 3DG-collagen is inducing caspase-3 activation through ER stress, which is dependent on upstream activation of ROS and p38 MAPK through upregulation of Nox4.

3DG-collagen induces ROS and apoptosis independent of RAGE signaling

When AGEs bind to their receptor, RAGE, ROS are released as a downstream byproduct [25]. To determine if 3DG-collagen is signaling via its interaction with RAGE, we investigated transcript levels of total RAGE. To our surprise, 3DG-collagen did not upregulate RAGE transcript levels (Figure 8A). As a control for the induction of RAGE we cross-linked collagen with MG, which is a well studied AGE precursor known to signal via RAGE [30,31]. We observed a significant induction of RAGE transcript levels in fibroblasts cultured on MG-collagen; 400%±12% upregulation of RAGE (Figure 8A, $p<0.002$). Additionally, treatment of MG-collagen with AG reduced the transcript levels of RAGE to 119%±13.3%, which confirms that MG can upregulate RAGE mRNA expression. To ensure that 3DG-collagen is not regulating the RAGE receptor post-transcriptionally we measured RAGE protein levels by Western blot. In contrast to the 238%±11.8% upregulation of RAGE protein in fibroblasts cultured on MG-collagen, 3DG-collagen did not upregulate RAGE protein expression in fibroblasts (107%±10.5%; Figure 8B). To further confirm the specificity of MG, AG abrogated the increase in RAGE protein expression (104%±8.2%) in fibroblasts cultured on MG-collagen (Figure 8B, $p<0.002$). This data suggests that 3DG-collagen is not upregulating RAGE at both the level of transcription or translation.

To further verify the absence of RAGE expression in 3DG-collagen signaling, RAGE was blocked using a blocking antibody

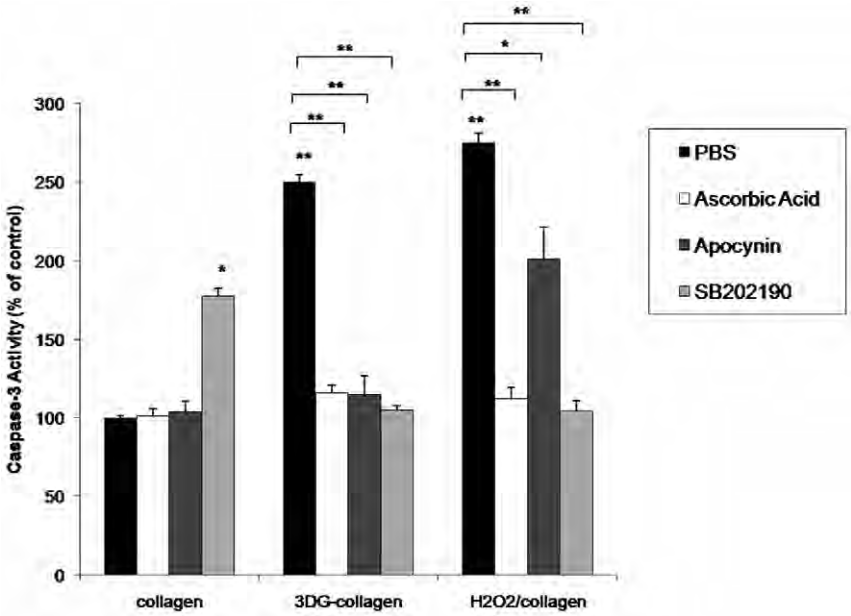

Figure 7. Inhibition of ROS, and p38 MAPK reduces caspase-3 cleavage induced by 3DG-collagen. Fibroblasts were pretreated with 100 μg/mL of ascorbic acid, 1 mM apocynin, or 10 μM SB202190 for 1 h and cultured on native collagen, 1 mM 3 DG-collagen, or treated with 50 μM H_2O_2 for 24 h. Treatment of fibroblasts with 50 μM H_2O_2 for 24 h was used as a positive control. 100 μg of whole cell lysate was assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean±SD (n = 3), **P<0.0002, *P<0.001.
doi:10.1371/journal.pone.0011093.g007

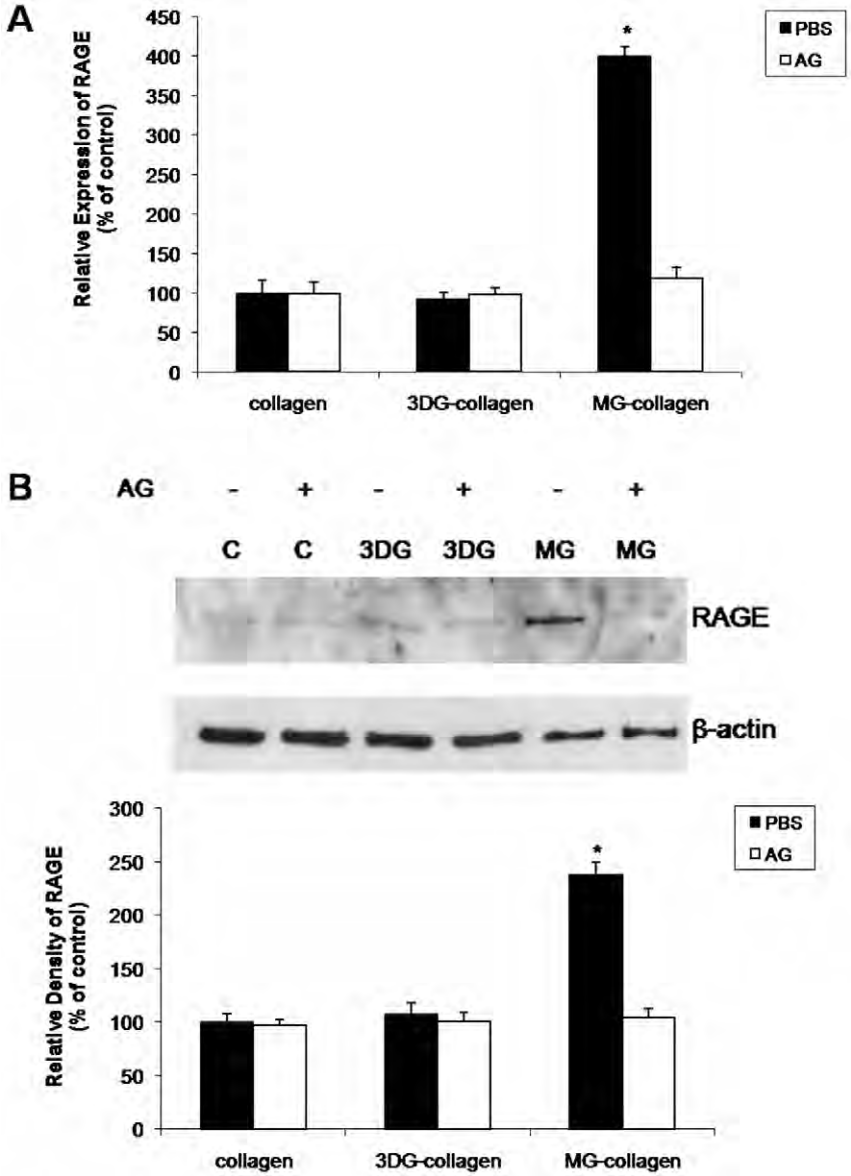

Figure 8. 3 DG-collagen does not induce the expression of RAGE. A, Fibroblasts were cultured on native collagen, 1 mM 3 DG-collagen, or 1 mM MG-collagen with or without 5 mM AG for 24 h. mRNA was analyzed for the expression of RAGE by real-time PCR. All transcripts were normalized to β-actin. **B**, Fibroblasts were treated as in **A** and analyzed for the expression of RAGE by Western blot. The bars correspond to the densitometric value of RAGE after normalization for β-actin. All comparisons are made against collagen treated with PBS. Data are mean ± SD (n = 3), *P<0.002.

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specific for the extracellular domain of RAGE, and the levels of ROS were quantified. Intriguingly, the levels of ROS in fibroblasts cultured on 3DG-collagen did not alter after blockade of RAGE. However, we observed the downregulation of ROS with the inhibition of RAGE binding in fibroblasts cultured on MG-collagen (Figure 9A, $p<0.001$). To further demonstrate that 3DG-collagen signaling was independent of RAGE, we investigated the expression of GADD153. Fibroblasts were pretreated with the RAGE blocking antibody and cultured on native collagen, 3DG-collagen, or MG-collagen; and the level of activated GADD153 was quantified. Blockade of RAGE in fibroblasts cultured on 3DG-collagen did not suppress the activation of GADD153, while GADD153 was suppressed after blockade of RAGE in fibroblasts cultured on MG-collagen (Figure 9B–C, $p<0.001$). Blockade of RAGE did not decrease the level of caspase-3 activity in fibroblasts cultured on 3DG-collagen, while suppression of caspase-3 activity was observed in fibroblasts pretreated with the RAGE antibody and cultured on MG-collagen (Figure 9D, $p<0.001$). These results suggest that 3DG-collagen is not signaling through the RAGE receptor as is observed with MG.

3DG-collagen activates the ER stress signaling cascade through α1β1 integrin

To delineate the receptor involved in activating the ER stress pathway by 3DG-collagen, we investigated α1β1 integrin collagen receptor. Previous data has demonstrated that fibroblasts have an increased adherence to 3DG-collagen, which is dependent on binding by α1β1 integrin [28]. Fibroblasts can change their binding affinity for 3DG-collagen, which may cause an overpro-

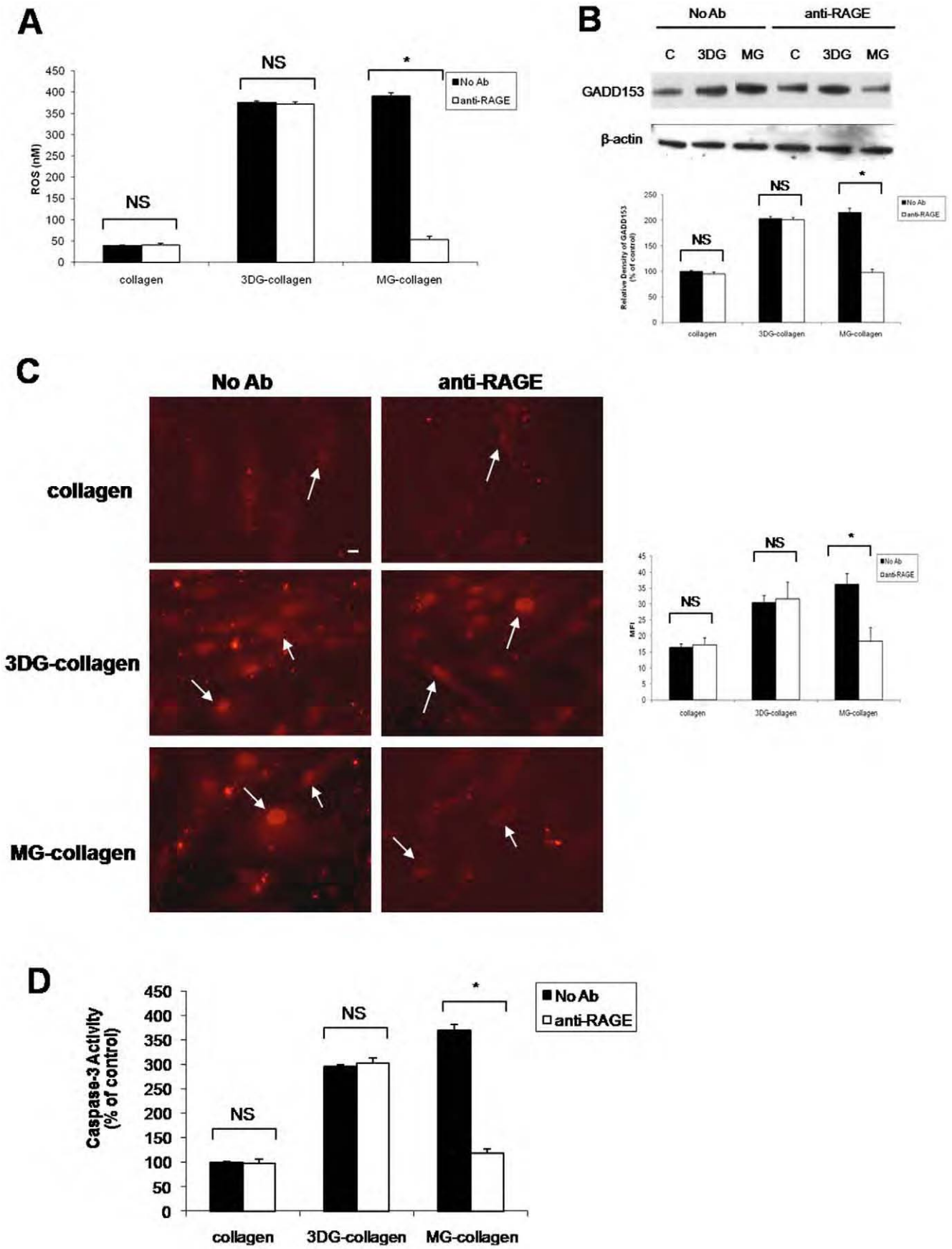
A
ROS (nM)
NS
NS
*
No Ab
anti-RAGE
collagen
3DG-collagen
MG-collagen
B
No Ab
anti-RAGE
C
3DG
MG
GADD153
β-actin
Relative Density of GADD153 (% of control)
C
No Ab
anti-RAGE
collagen
3DG-collagen
MG-collagen
MFI
D
Caspase-3 Activity (% of control)

Figure 9. Inhibition of RAGE does not alter the induction of ER stress pathway in fibroblasts cultured on 3 DG-collagen. A, Fibroblasts were pretreated with or without the blocking antibody anti-RAGE (10 μg/μL) for 1 h and cultured on native collagen, 1 mM 3 DG-collagen, or 1 mM MG-collagen for 24 h and analyzed for the production of ROS. Fibroblasts were incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. **B**, Western blot of GADD153 expression after inhibition of RAGE. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. **C**, GADD153 localization in the nucleus was analyzed by immunofluorescence using a Cy3-conjugated secondary antibody. Images were taken at 40× magnification on an epi-fluorescence microscope and the MFI of ten nuclei was processed by ImageJ. Arrows indicate nuclei containing GADD153. Scale bar represents 10 μm. **D**, Caspase-3 activity analyzed according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. Data are mean±SD (n = 3), *P<0.001.
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duction of ROS resulting in increased caspase-3 activation. To verify the role of α1β1 integrin on the ER stress signaling pathway, fibroblasts were pretreated with blocking antibodies against either β1 or α1 integrin and the level of ROS was quantified. α5 integrin, the alpha subunit responsible for binding fibronection, was used as a negative control. Neutralization of both β1 and α1 integrin reduced the production of ROS in fibroblasts cultured on 3DG-collagen to that seen in fibroblasts cultured on native collagen, while neutralization of α5 integrin did not affect the production of ROS (Figure 10A, $p<0.001$). We next investigated the effect of β1 and α1 integrin neutralization on the expression of GADD153 in fibroblasts cultured on native collagen or 3DG-collagen for 24 h. Blockade of both β1 and α1 integrins suppressed the activation of GADD153 as seen by decreased protein expression and nuclear localization (Figure 10B–C, $p<0.001$). Moreover, neutralization of β1 and α1 integrin in fibroblasts cultured on 3DG-collagen reduced the activity of caspase-3 to that observed in fibroblasts cultured on native collagen (Figure 10D, $p<0.001$). These results suggest that the increased binding affinity of α1β1 integrin to 3DG-collagen causes the overproduction of ROS, which in turn leads to increased GADD153 activation and cleavage of caspase-3.

Discussion

Previous results from our laboratory demonstrated a role for the ER stress pathway in 3DG-collagen signaling; however, the exact signaling mechanism of how 3DG induced ER stress remained elusive [28]. The goal of the present study was to further investigate the induction of caspase-3 activation as an early marker of apoptosis and the signaling events required in fibroblasts cultured on 3DG-collagen. The present study demonstrated that 3DG-collagen induced caspase-3 activation of dermal fibroblasts through activation of GADD153 via induction of ROS and p38 MAPK. Initial studies showed an increase in the apoptotic signaling cascade in fibroblasts cultured on 3DG-collagen, which was confirmed by increases in caspase-3 activity (Figure 1). The level of caspase-3 activity was found to be decreased in fibroblasts cultured on 3DG-collagen and treated with the inhibitors AG and meglumine. Furthermore, we confirmed that 3DG-collagen was inducing caspase-3 activity through induction of ER stress by using an inhibitor of ER stress, salubrinal (Figure 1).

In addition to examining the level of caspase-3 activity, we further investigated the role of ER stress and GADD153 in 3DG-collagen signaling. GADD153 is a marker for misfolded proteins in the ER [37,43]. It is well documented that ER stress can induce GADD153, which can lead to apoptosis of the cell [33,34,37,43,46–48]. 3DG-collagen induced a significant increase in the level of GADD153, which could be inhibited with AG and meglumine. We confirmed that 3DG-collagen is activating GADD153 through induction of ER stress as salubrinal abolished the 3DG-collagen-induced expression of GADD153 (Figure 2).

To further delineate the signaling mechanism by which 3DG-collagen activates caspase-3, we investigated the expression of ROS. Although ROS are seen as beneficial to the cell during the initial stages of wound healing, chronic activation of ROS have been shown to be stressors on the cell leading to apoptosis [38,54]. ROS have been shown to induce apoptosis through many pathways, one of which includes ER stress [34,35,41]. Additionally, circulating AGEs are known to induce the expression of ROS in many different cell types including neuronal and endothelial cells [15–20]. Therefore, we investigated the role of ROS in 3DG-collagen-induced caspase-3 activation in fibroblasts. 3DG-collagen produced ROS, which was attenuated with the addition of the antioxidant ascorbic acid. Inhibitors of 3DG-collagen, AG and meglumine, reduced the level of ROS to that observed in fibroblasts cultured on native collagen suggesting that 3DG is directly responsible for the ROS produced in the fibroblast (Figure 3). To determine the source of increased ROS in fibroblasts cultured on 3DG-collagen we focused our attention to the activation of Nox4. Nox4 has been shown to be highly expressed in fibroblasts [38,39] and Nox4 has been shown to induce ROS through integrin and growth factor signaling [38]. Fibroblasts cultured on 3DG-collagen produced a significant upregulation in the expression of Nox4 both transcriptionally and translationally (Figure 4A–B). This effect could be inhibited with AG and meglumine suggesting that 3DG-collagen specifically induced Nox4 expression. To determine the role of Nox4 in 3DG-collagen-induced ROS, the Nox inhibitor apocynin was used. Pretreatment of fibroblasts with apocynin reduced the level of intracellular ROS to that observed in fibroblasts cultured on native collagen suggesting that Nox4 is responsible for the production of ROS by 3DG-collagen (Figure 4D).

During times of cellular stress, ROS can induce apoptosis via p38 MAPK activation [35,37,43,44]. p38 MAPK is a stress-activator kinase and has been shown to be an upstream mediator of GADD153 activation [35,37,43,44]. 3DG-collagen-induced phosphorylation of p38 MAPK was found to be dependent on upstream ROS production by Nox4 as the reversal of p38 MAPK phosphorylation occurred when fibroblasts were pretreated with the antioxidant ascorbic acid and the Nox inhibitor apocynin (Figure 5). Moreover, inhibition of ROS and p38 MAPK reduced the level of GADD153 activation and caspase-3 activity of the cell. These data suggest that 3DG-collagen induces an apoptotic signaling cascade through the ER stress pathway, which is dependent on ROS and p38 MAPK activation via Nox4 (Figures 6–7).

It has been well documented that production of intracellular ROS by AGEs induces apoptosis through interaction with its receptor RAGE [19,23–25]. Unlike MG-collagen, a well-studied AGE precursor known to upregulate RAGE, 3DG-collagen did not upregulate the RAGE receptor both transcriptionally or translationally (Figure 8A–B). To provide further evidence that RAGE is not responsible for the ROS-induced caspase-3 activity by 3DG-collagen, a blocking antibody was employed to inhibit the extracellular domain of RAGE. We observed no changes in the levels of ROS, GADD153 expression, or caspase-3 activity when RAGE was blocked suggesting that 3DG-collagen is not signaling through the RAGE receptor (Figure 9A–D). We have previously

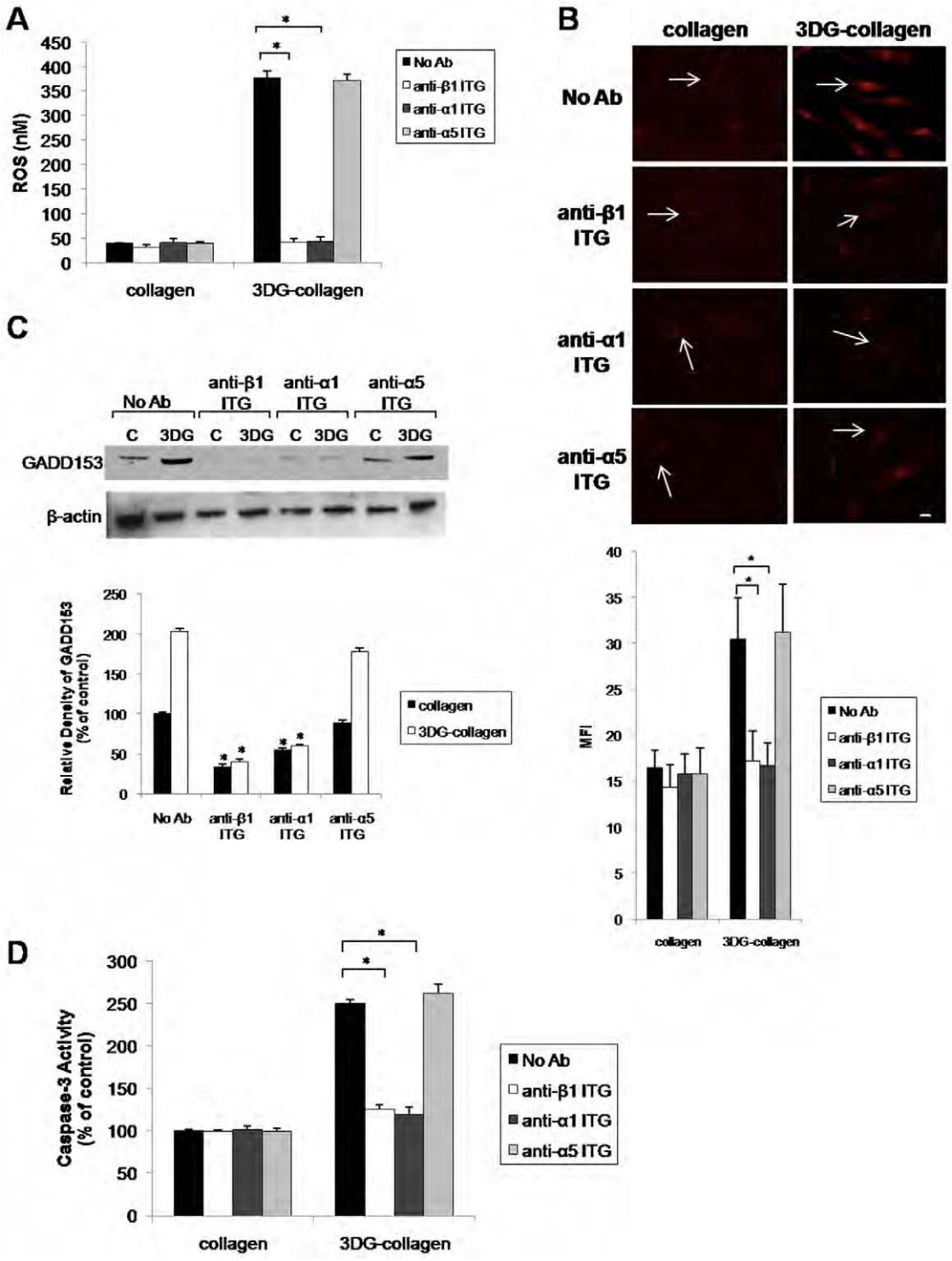

Figure 10. Effect of neutralization of α1β1 integrin on 3 DG-collagen-induced ER stress pathway. A, Fibroblasts were pretreated with or without the blocking antibodies anti-β1 ITG, anti-α1 ITG, and anti-α5 ITG (10 μg/μL) for 30 min and cultured on native collagen, or 1 mM 3 DG-collagen, for 24 h and analyzed for the production of ROS. Fibroblasts were incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. **B**, GADD153 localization in the nucleus was analyzed by immunofluorescence using a Cy3-conjugated secondary antibody. Images were taken at 40× magnification on an epi-fluorescence microscope and the MFI of ten nuclei was processed by ImageJ. Arrows indicate nuclei containing GADD153. Scale bar represents 10 μm. **C**, Western blot of GADD153 expression after neutralization of β1, α1, and α5 integrins. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. **D**, Caspase-3 activity detected using the Caspase-3 Colorimetric Correlate Assay. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean±SD (n = 3), *P<0.001.

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demonstrated that 3DG-collagen tightly binds to the collagen integrins α1β1, which causes the cell to become static and incapable of migration [28]. Therefore, we proposed that by changing the binding affinity of the fibroblast to collagen, integrins are causing the induction of intracellular ROS, which leads to oxidant stress and caspase-3 activation. We demonstrated that neutralization of the collagen receptors integrin α1 and β1, abrogated the production of ROS by 3DG-collagen (Figure 10A). Additionally, blockade of integrin α1 and β1 reduced the expression of GADD153 and the activation of caspase-3 to that seen in fibroblasts cultured on native collagen (Figure 10 B–D). As a negative control fibroblasts were pretreated with the fibronectin receptor integrin α5, which did not alter the levels of ROS, GADD153 expression, or caspase-3 cleavage (Figure 10). These data suggest that α1β1 integrin on the dermal fibroblast is the receptor responsible for 3DG-collagen-induced caspase-3 activation.

With the growing knowledge of AGEs and their precursors, it is becoming clearer that each AGE and precursor could signal differently depending upon not only the cell type but also the state of the AGE, whether circulating or protein bound. This is believed to be the first documented report to conclusively demonstrate that 3DG-collagen signals independently of RAGE to induce ROS and activate ER stress-induced caspase-3 activation. Because of the number of different AGEs, it is becoming increasingly important to understand how they signal within the cell in order to provide better therapeutics. In addition to the use of AG we studied a new promising therapeutic, meglumine, which decreased caspase-3 activation in the cell by abolishing the production of ROS and activation of GADD153. Additionally, meglumine was shown to reverse the 3DG-collagen mediated effects on fibroblasts by promoting fibroblast migration and proliferation, and increasing ECM production [28,29]. With the growing number of elderly and diabetic patients, the number of people suffering from diabetic complications associated with AGE formation will continue to increase; therefore, it is vital to gain a better understanding of not only 3DG signaling but of all AGE signaling pathways.

Methods

This study was approved by the Internal Review Board of Drexel University for human studies.

Collagen Coating of Cultured Dishes

Acid extracted type I collagen (95–97% COL1A1; 3–5% COL3A1) from human skin was purchased from Stem Cell Technologies (Vancouver BC, Canada). The collagen was diluted in PBS to a final concentration of 0.067 mg/ml. The diluted collagen was added to the tissue culture dish for 2 h at 37°C as previously described [28]. The culture dish was washed 3 times with 5 mL of sterile PBS to remove any nonadherent collagen from the dish. To modify the matrices, collagen coated dishes were incubated overnight with 1 mM 3 DG or 1 mM MG, and/or 5 mM AG, which was added simultaneously to the collagen. Unincorporated 3 DG, MG, or AG was removed by gently washing the collagen three times with sterile PBS prior to plating with fibroblasts. For treatment with H_2O_2, fibroblasts were cultured on native collagen followed by the addition of 50 μM H_2O_2 for 24 h.

Tissue Culture

Normal human dermal fibroblasts from individuals (GM06120, GM00498, GM04190) aged 3–85years old (less than passage 15) were purchased from the Coriel Institute (Camden, NJ). Unless otherwise noted, fibroblasts were seeded onto native collagen and 3DG-collagen coated dishes and cultured until 70% confluent in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% dialyzed FBS and 1% penicillin/streptomycin.

Chemicals and Antibodies

Salubrinal was purchased from Calbiochem (La Jolla, CA). SB202190 and ascorbic acid were purchased from Sigma (St. Louis, MO). 6-Carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Cell Biolabs Inc. (San Diego, CA). H_2O_2 was purchased from Fisher Scientific (Pittsburgh, PA). Apocynin, the monoclonal antibody against GADD153, and polyclonal antibodies against Nox4 and RAGE were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against phospho-p38 MAPK, total p38 MAPK, and β-actin were purchased from Cell Signaling Technologies (Danvers, MA). Secondary antibodies were purchased from Jackson Labs (West Grove, PA). Meglumine-HCl was a kind gift from Dynamis Therapeutics, Inc (Jenkintown, PA).

Inhibition of ROS, p38 MAPK, GADD153, and RAGE

For inhibition studies, fibroblasts were cultured until 70% confluent, trypsinized, preincubated for 1 h with or without the antioxidant ascorbic acid (100 μg/mL), the ER stress inhibitor salubrinal (40 μM), the p38 MAPK inhibitor SB202190 (10 μM), the Nox4 inhibitor apocynin (1 mM), or the blocking antibody against RAGE (10 μg/μL), and replated onto collagen, 3DG-collagen, and MG-collagen coated dishes or treated with 50 μM H_2O_2 for 24 h in DMEM containing 1% FBS and 1% penicillin/streptomycin. The concentrations of inhibitors are similar to doses used in previously published studies [8,21,22,55–58].

Integrin Neutralization

Integrin neutralization assays were performed using blocking antibodies against integrins β1, α1, and α5 (Santa Cruz, CA) according to [59] to determine the involvement of integrins in ROS production, GADD153 activation, and caspase-3 cleavage. 70% confluent cells were suspended in 1% FBS-DMEM, incubated with antibodies (10 μg/μL) for 30 min at 37°C and then plated on chamber slides coated with collagen or 1 mM 3DG-collagen for 24 h.

SYBR Green Quantitative RT-PCR

Cells were harvested and RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's protocol. To verify expression of Nox4 and RAGE; 2.0 μg of total RNA was reverse-transcribed using Superscript-III reverse transcriptase (Invitrogen Carlsbad, CA), according to manufacturer's protocol. Transcripts were quantified using SYBR green PCR amplification (Qiagen). All mRNA transcripts were normalized to β-actin expression. The following primers were employed to detect transcripts of interest:

Nox1-forward: 5′-TTCACCAATTCCCAGGATTGAAGTG-GATGGTC-3′

Nox1-reverse: 5′-GACCTGTCACGATGTCAGTGGCCTT-GTCAA-3′;

Nox2-forward: 5′-AACGAGCAGGCGCTGGCGTCC-3′

Nox2-reverse: 5′-GCTTGGGCTCGATGGGCGTCCACT-3′;

Nox4-forward: 5′-CTGGAGGAGCTGGCTCGCCAACGAAG-3′

Nox4-reverse: 5′-GTGATCATGAGGAATAGCACCACCA-CCATGCAG-3′;

RAGE-forward: 5′-CAGGACCCTGGAAGGAAGCA-3′

RAGE-reverse: 5′-TGATGGATGGGATCTGTCTGTG-3′;

β-actin-forward 5′-TTGCCGACAGGATGCAGAA-3′
β-actin-reverse 5′-GCCGATCCACACGGAGTACTT-3′.

Immunofluorescence

Cells cultured in chamber slides for 24 h at 30% confluency were fixed in 4% paraformaldehyde for 10 min. Cells were incubated in a 1:50 dilution of GADD153 or Nox4 antibodies and incubated in a humid chamber at room temperature for 60 min. The cells were washed 3 times with PBS and then stained with Cy3 or Cy2 secondary Ab (1:50 dilution) (Jackson Labs) in a humid chamber at room temperature for 40 min. Cells were washed 3 times with PBS and mounted with DAPI. Images were viewed with an epi-fluorescence microscope at 40× magnification. Ten images from each preparation were taken. For GADD153 analysis, the mean fluorescence intensity (MFI) of the nuclei of each cell was calculated using ImageJ.

Western blot

Cells were harvested and protein was extracted using 100 μL of cell lysis buffer supplemented with 0.3% PMSF and proteinase and phosphatase inhibitors. 100 μg of protein from each sample was size fractionated on 10% SDS PAGE gels (Invitrogen) for 60 min at 180 volts. The proteins were transferred to PVDF membrane and the membrane blocked with 5% skim milk. The PVDF was probed with an antibody directed against either GADD153 (1:200), Nox4 (1:200), RAGE (1:500), phospho-p38 MAPK (1:1000), total p38 MAPK (1:1000), or β-actin (1:1000). The membrane was washed with TBS-Tween to remove any unbound proteins and incubated with the secondary antibody, goat-anti-rabbit-HRP (1:2000). The signal was developed with SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL).

Reactive Oxygen Species Assay

Cells were cultured in a 96-well collagen or 3DG-collagen-coated plates for 24 h. As a positive control, cells were cultured on native collagen and treated with 50 μM H_2O_2 for 24 h. The level of ROS was then quantified according to the manufacturer's protocol (Cell Biolabs, Inc., San Diego, CA). The cells were treated with 1× DCFH-DA solution in DMEM for 30 min at 37°C, washed with PBS, and the assay was terminated by the addition of 2× Cell Lysis Buffer. 150 μL of the lysis mixture was added to a 96-well plate and the fluorescence of the lysate was measured at 480 nm/530 nm on a Fluoroskan Ascent FL (LabSystems, Beverly, MA).

Caspase-3 Assay

Cells were harvested and lysed in cell lysis buffer as described above. Whole cell lysates were combined with Caspase-3 substrate reaction buffer and incubated for 3 h at 37°C and the absorbance was measured at 450 nm with a plate reader according to the manufacturer (Assay Designs, Ann Arbor, MI). Background readings from cell buffers and substrates were subtracted from the sample readings. A standard curve was used to calculate the increase in caspase-3 activity.

Statistical Analysis

The results are mean±SD. The resulting data were subjected to either a two-tailed paired *t*- test for comparison between two groups, or a one-way ANOVA for comparison between multiple groups followed by Tukey's post-hoc test. A P value$<$0.05 was considered significant.

Author Contributions

Conceived and designed the experiments: DTL CMA. Performed the experiments: DTL. Analyzed the data: DTL. Contributed reagents/materials/analysis tools: DTL CMA. Wrote the paper: DTL CMA.

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MEMORANDUM

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Total pediatrics / Immunology
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FROM: Sreekant Murthy. Ph.D.
Sreekant Murthy, ~~Ph.D.~~
Vice Provost for Regulatory Research Compliance

RE: Protocol - Modulation of Fibrosis in Scleroderma by 3-Deoxyglucosone
Project No.: 1041730
Protocol No.: 16793

DATE: November 10, 2008

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