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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 $\alpha 1\beta 1$ 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 estimated that the work in Specific Aim 1, would take approximately 2 years to complete. This aim is involved in identifying the signaling pathway or pathways within the fibroblasts that is responsible for the decrease in extracellular matrix expression in response to the 3DG modified collagen matrices. At the time of writing the application, we believed that the ERK1/2 pathway was involved in the response of fibroblasts to the 3DG-modified collagen matrices and we have conclusively demonstrated that the ERK1/2 pathway is one of the major pathways involved in reduced collagen expression. However, we have identified alterations in the p38 MAP kinase pathway as well. We are very close to finishing this aim and have a couple of experiments the will be completed by the end of May, 2009.

We have now received a total of 12 of the 16 paired cell lines that we estimated we require for the experiments proposed from Carol Feghali-Bostwick, PhD at Pittsburgh University. These paired cell lines were taken from involved and uninvolved skin from scleroderma patients. Involved skin is where the clinically affected fibrotic lesion and the uninvolved skin comprises of a non-fibrotic/clinically unaffected areas, however although this latter area is considered to be clinically normal but it is not normal in regards to gene expression. We have also purchased a total of 10 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.

1. 3DG-Collagen Decreases Fibroblast ERK1/2 Phosphorylation

Phosphorylation of ERK1/2 (also called p42/p44 MAP kinase) is important in intracellular signaling and plays a crucial role in promoting cell proliferation and differentiation (1-3). The ERK proteins are found at sites in the signaling cascades where the signals converge and activate ERK1/2, which can regulate cytoskeletal remodeling, cell migration, and promote cell cycle progression (4-6). We found that normal fibroblasts cultured on 3DG-collagen had ERK1/2 phosphorylation decreased to 55% $\pm$ 2.12% ($P < 0.007$) compared to normal fibroblasts cultured on native collagen (Figure 1). Likewise with the SSc fibroblasts, we found that there was a significant reduction in the phosphorylation of ERK1/2. ERK1/2 phosphorylation was decreased to 63.6% $\pm$ 3.9% of SSc fibroblast values cultured on native collagen ($P= 0.0064$, Figure 1).

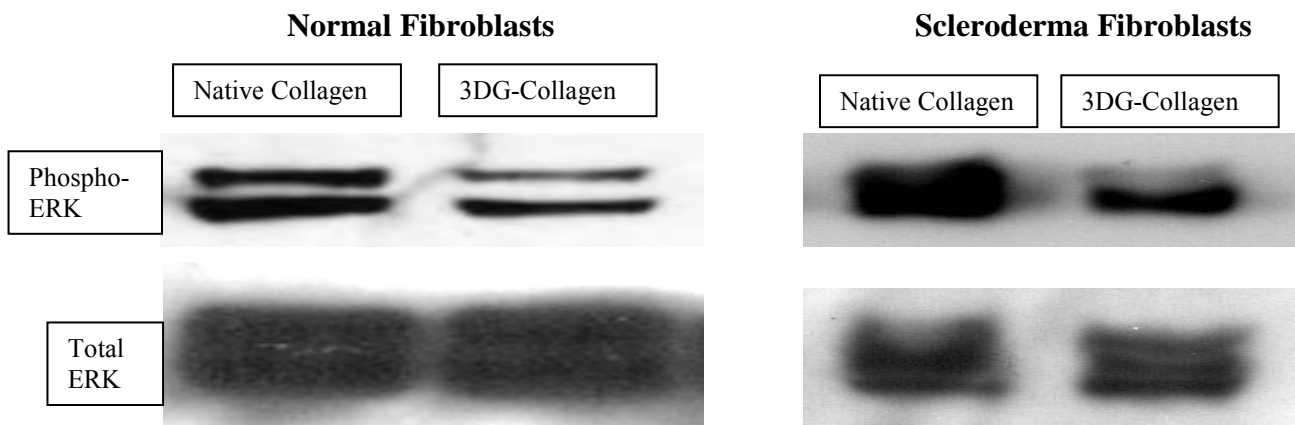**Normal Fibroblasts**   **Scleroderma Fibroblasts**

Figure 1. Phosphorylation of ERK1/2 (p42/p44) in normal and SSc fibroblasts cultured on 3DG-collagen. Normal and SSc fibroblasts were cultured on native or 3DG-collagen for 72 h. Protein was extracted from the fibroblasts and size fractionated on polyacrylamide gels, transferred to PVDF membrane and probed for ERK1/2 using a specific antibody against phosphorylated ERK1/2. The densities of the bands were quantified by ImageJ and corrected for total ERK protein. Both normal and SSc fibroblasts decreased ERK1/2 phosphorylation when cultured on 3DG-collagen matrices; normal fibroblasts to 55% $\pm$ 2.12% (P < 0.007) and SSc fibroblasts to 63.6% $\pm$ 3.9% (P= 0.0064).

2. **MEK1/2 phosphorylation is decreased with 3DG-collagen.**

As ERK1/2 signals from MEK1/2, we wanted to determine if there was also an alteration in the phosphorylation of MEK1/2. After the bands were corrected for total MEK1/2 protein, we found that the averaged MEK1/2 phosphorylation was decreased to 61.9% when SSc fibroblasts. We found also that normal fibroblasts also had reduced MEK1/2 phosphorylation but 3DG-collagen did not decrease the phosphorylation of MEK1/2 significantly and it was found to be 90% of that observed on native collagen. The differences with SSc fibroblasts on 3DG-collagen was found to be statistically significant (P = 0.005; Figure 2).
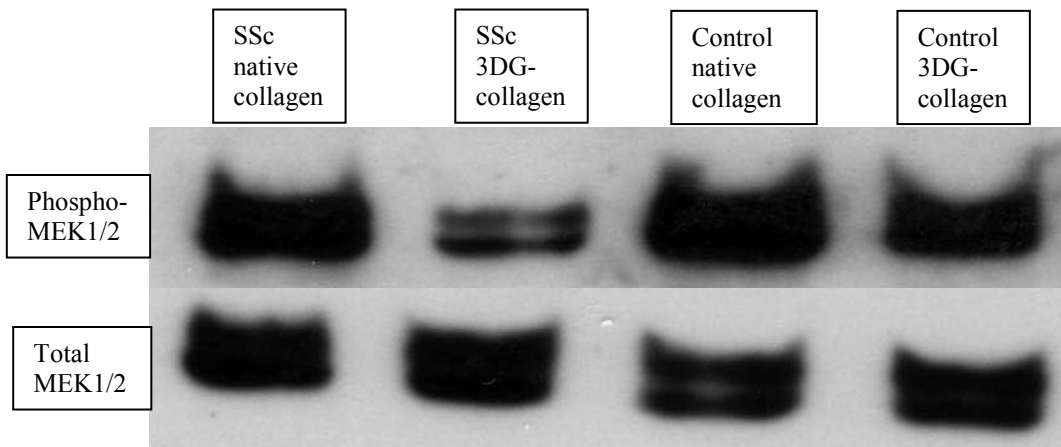
Figure 2. Phosphorylation of MEK1/2 in normal and SSc fibroblasts cultured on 3DG-collagen. Normal and SSc fibroblasts were cultured on native or 3DG-collagen for 72 h. Protein was extracted from the fibroblasts and size fractionated on polyacrylamide gels, transferred to PVDF membrane and probed for MEK1/2 using a specific antibody against phosphorylated MEK. The densities of the bands were quantified by ImageJ and corrected for total MEK protein. SSc fibroblasts significantly decreased MEK1/2 phosphorylation when cultured on 3DG-collagen matrices to 61.9% $\pm$ 2.1% of native collagen (P= 0.005).

We further confirmed the Western blot analysis with staining of fibroblasts cultured on native collagen vs. 3DG-collagen and staining for ERK1/2 and MEK1/2 protein (Figure 3).

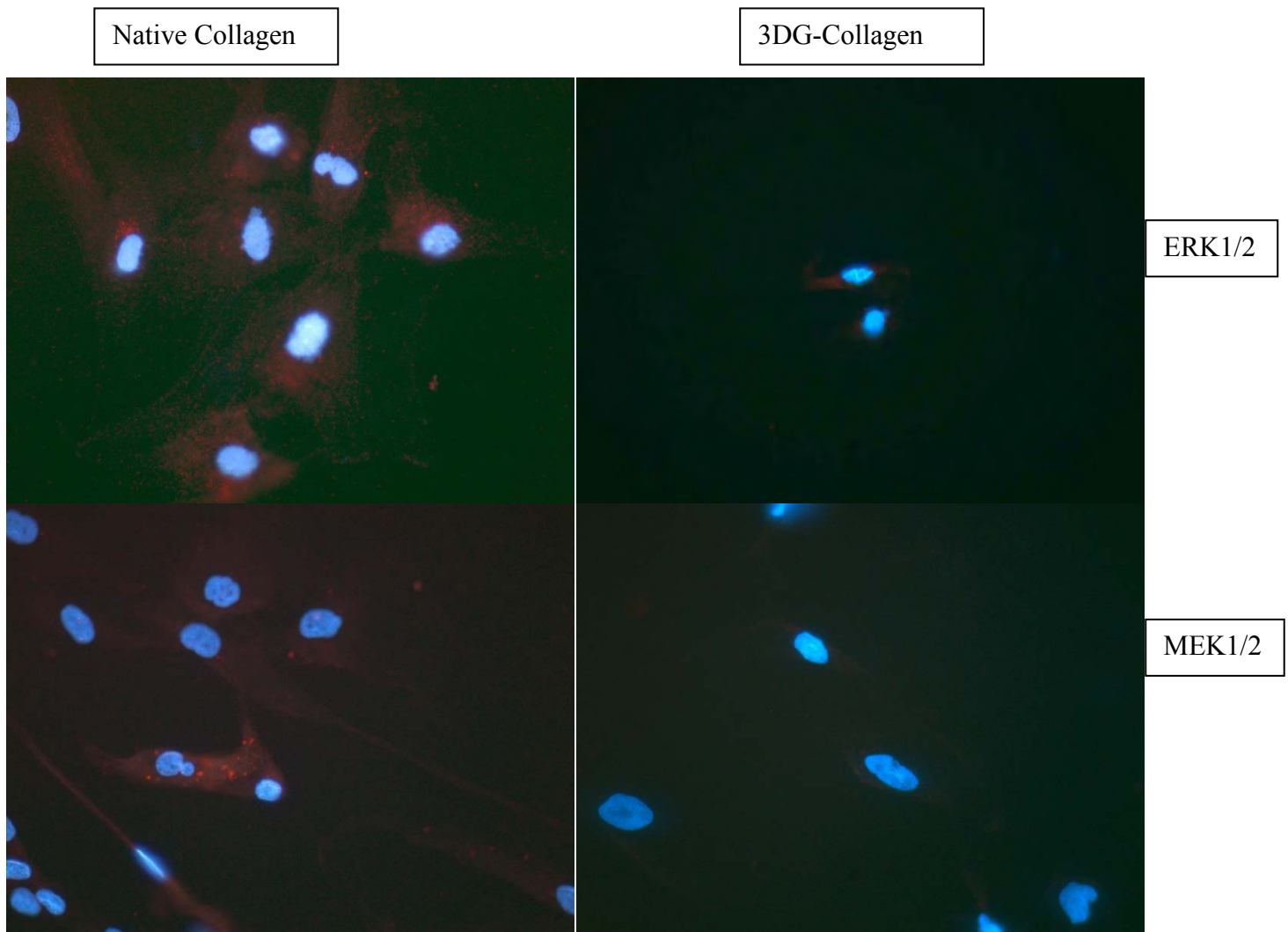**Figure 3. Histological analyses of ERK1/2 and MEK1/2 in fibroblasts cultured on native and 3DG-collagen.** Fibroblasts were cultured on native or 3DG collagen and stained for ERK1/2 or MEK1/2, counterstained with DaPI to denote the nuclei (blue) and photographed. Note the intense staining of both ERK1/2 and MEK1/2 on native collagen, whereas there was very little staining for both ERK1/2 and MEK1/2 on the 3DG-collagen.

3. Electrophoretic Mobility Shift Assays.

We know from previous work from my laboratory, the region spanning -174 to +1 in the collagen promoter is the most active part of the promoter of the COL1A1 gene (7). It contains two NF-1/Sp1 switch elements. The proximal NF-1/Sp1 switch element spans -107 to -64 and the distal switch site spans -174 to -107 bp upstream from the transcription start site. Therefore, we designed primers that isolated each of the switch elements individually. We found that the distal part of the promoter affected collagen expression and was sensitive to the 3DG-collagen (Figure 4). This is in keeping with our previously published data that demonstrated that the proximal NF-1/Sp1 had almost no promoter activity (7) and that the distal NF-1/Sp1 switch element modulated collagen expression. Note that there is no binding of protein to the distal promoter region with the nuclear extract from fibroblasts cultured on 3DG-collagen as evidenced by lack of a band. This suggests that there is a dramatic reduction in transcription factors, which we confirm in Figure 5.
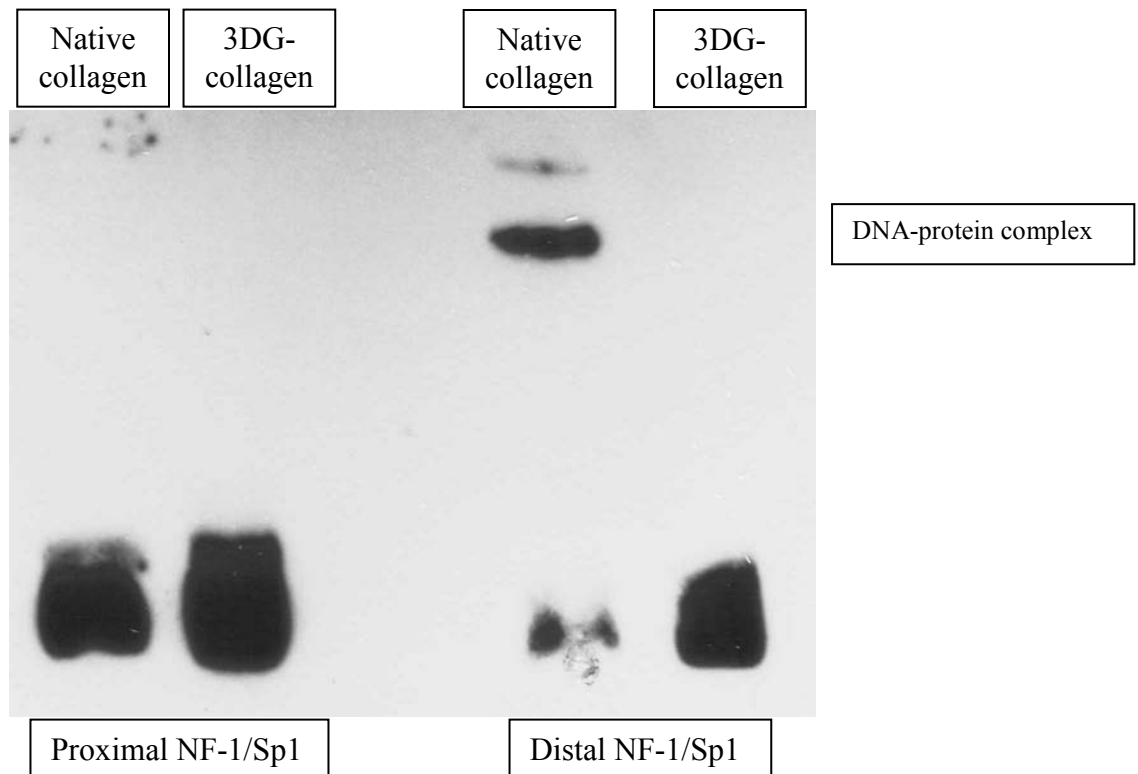

Figure 4. The Distal NF-1/Sp1 Switch Element is Involved in Decreased Collagen Expression observed in SSc Fibroblasts Cultured on 3DG-collagen. SSc fibroblasts were cultured on native or 3DG-collagen. Nuclear extract was isolated and incubated with biotin-labeled distal or proximal regions of the collagen promoter. The DNA-protein complexes were size fractionated on polyacrylamide gels, transferred to PVDF membrane and probed with an anti-biotin antibody. We found that the distal NF-1/Sp1 switch element was affected by the 3DG-collagen as noted by the band shift. This band shift was not observed with nuclear extract from fibroblasts cultured on 3DG-collagen or with the proximal NF-1/Sp1 switch site.

4. Transcription Factor Analyses.

We went on to investigated the transcription factors that are the most important for collagen expression in SSc; Sp1, c-fos, and c-myc. We found that 3DG collagen decreased Sp1 transcription levels and Sp1 activity. Fibroblasts were cultured on 3DG-collagen or native collagen for 24 h and mRNA was purified, made into cDNA and Sp1, c-fos, and c-myc levels were assessed. We found that Sp1 transcripts were decreased by 88.7% (P < 0.0001), c-fos was increased by 6% (not significant), and c-myc mRNA transcripts did not

change (not significant). As Sp1 transcripts were decreased, we also investigated the transcription factor Sp3 by real-time PCR as we had previously demonstrated that Sp1 and Sp3 and able to interact to affect collagen gene regulation (8). We also found that Sp3 transcript levels were decreased by the 3DG-collagen; however, this decrease was modest (7%) and not statistically significant. We found that the changes to the activity levels of these proteins were not reflected by the alterations in the mRNA transcript numbers. This may be due to the increased degradation of these proteins in response to 3DG-collagen rather than alterations to transcription factor mRNA levels. We measured the activity of the proteins by ELISA assays in SSc fibroblasts and found that all the activities of all three transcripts were decreased; Sp1 activity was decreased by 37% (P = 0.0008), c-fos by 30% (P = 0.002)and c-myc by 17% (P = 0.01)(Figure 5).
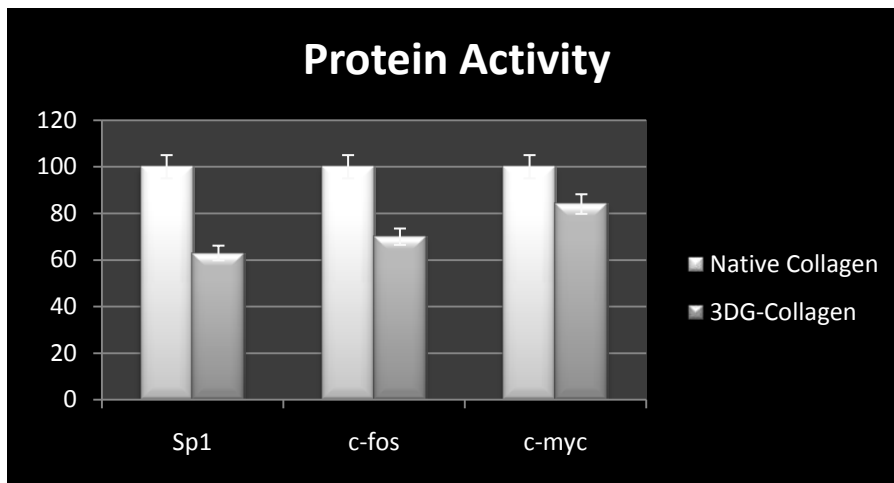

Figure 5. Transcription factor activity for Sp1, c-myc, and c-fos is decreased in fibroblasts cultured on 3DG-collagen. SSc fibroblasts were cultured on native or 3DG-collagen. Nuclear extract was isolated and Sp1, c-fos, and c-myc activities were measured by ELISA according to the manufacturer's recommendations. We found the activities of all three transcripts that were measured were decreased on 3DG-collagen; Sp1 activity was decreased by 37% (P = 0.0008), c-fos by 30% (P = 0.002) and c-myc by 17% (P = 0.01). These experiments have been performed 3 independent times and the values have been compiled.

   5. **DNA Affinity Precipitation.**
We had originally planned to performed DNA affinity purifications; however, it became apparent to us while we were analyzing the electrophoretic mobility shift assays that no protein was binding to the region that held the highest transcriptional rate for type I collagen when fibroblasts were cultured on 3DG collagen (Figure 4). We believe that this is due to the substantial depression of transcription factor activity (Figure 5) as we saw a complete abolishment of the shifted band. If we had observed a slight shift remaining in the DNA protein complex in Figure 4, we would have considered that another transcription factor has bound to this region and is inhibiting the expression of the collagen gene, however, we did not see this and therefore that no transcription factors are binding to this region when fibroblasts are cultured on 3DG-collagen.

Additional Experiments:
We have performed further experiments to try to elucidate the changes in signaling when fibroblasts are cultured on 3DG-collagen and how this translates to the decreased collagen expression. The observations of decreased collagen expression in SSc fibroblasts cultured on 3DG-collagen cannot be fully accounted for with just the decreased transcription factors through the ERK1/2 pathway, therefore we investigated other signaling pathways from the integrin receptor. These following observations further define the role for 3DG

in t he r educed e xpression of c ollagen w ithin t he s kin but a lso i mplicate t he r ole of 3D G i n di abetic complications such as chronic wounds.

6. Fibroblasts adhere more strongly to 3DG-collagen

We wanted to determine the binding strength of the fibroblast to the collagen fiber as this also induces feedback signals for collagen expression through the ERK pathway. Kinetic analyses have demonstrated that weakly adhesive surfaces induce fibroblasts to poorly adhere to the collagen and likewise, strongly adhesive surfaces can immobilize fibroblasts due to disruption of the cell-substratum attachments (9). Previous work in our laboratory demonstrated that there is decreased proliferation of fibroblasts when cultured on 3DG-treated collagen (see the Annual Report for 2007 – 2008). Therefore, we measured the fibroblast's adhesive strength to the 3DG-collagen using a jet wash adhesion assay. Figure 6E is a schematic representation of the number of cells binding to native collagen, 3DG-treated collagen, and aminoguanidine/3DG-treated collagen. Fibroblasts seeded onto native collagen begin to attach within 3 h, and this attachment is difficult to disturb by mechanical disruption (10). After 3 h a jet wash assay was performed and ten random fields of view were captured at 10X magnification and the numbers of cells in each field were counted. We found that an average of 280 cells/field remained attached on native collagen (Figure 6A). In contrast, we observed an average of 353 cell/field remained on 3DG-collagen, representing an increase in cell adherence by $26\% \pm 1.41\%$ (Figure 6B; $P < 0.0001$). Additional experiments with 5mM AG, which chelates 3DG prior to its reaction with the collagen, did not affect fibroblast binding to the collagen and an average of 276 cells/field remained with aminoguanidine (Figure 6C) and native collagen compared to 283 cells/field with aminoguanidine and 3DG-collagen Figure 6D). Figure 6E is a graphical representation of the number of cells that adhere with each condition after the jet wash assay.
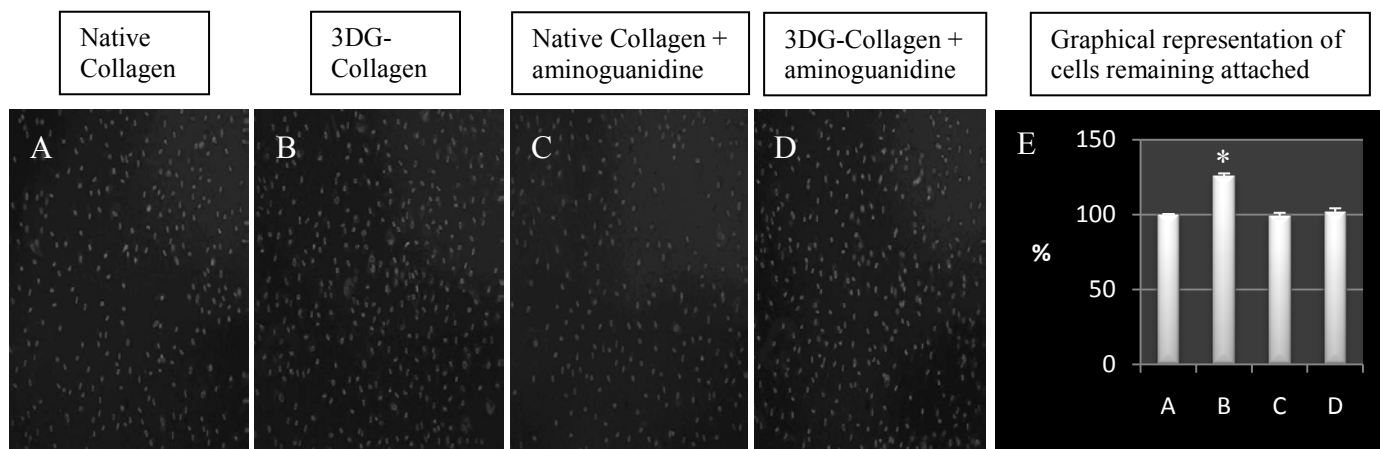
Native Collagen	3DG-Collagen	Native Collagen + aminoguanidine	3DG-Collagen + aminoguanidine	Graphical representation of cells remaining attached

Figure 6. Effect of 3DG on Fibroblast Adhesion to Collagen. Normal fibroblasts were seeded on native collagen (A), collagen cross-linked by 1mM 3DG (B), native collagen + aminoguanidine (C), or 3DG cross-linked collagen and aminoguanidine (D) and allowed to attach for 3h. Collagen adhesive strength was measured by counting the number of cells remaining after a jet wash assay according to Arnesen et al 2006 (10). Cell numbers from 10 random images were counted and averaged and this experiment was performed on 3 separate occasions. (A-D) Show a representative image of the numbers of fibroblasts remaining after the jet wash assay. (E) This is a graphical representation of the number of cells adhering to the collagen after the jet wash assay. Fibroblasts were found to adhere $26\% \pm 1.4\%$ more efficiently to the 3DG-collagen (B) than that which was found to adhere to the native collagen (A) *$P < 0.03$. The effect of 3DG was abolished with aminoguanidine and the number of cells was comparable to native collagen indicating that 3DG directly changes the adhesive properties of the fibroblast to the collagen (C & D). All images were taken at 10X magnification.

These f indings could a ccount f or t he de creased pr oliferation of f ibroblasts w hen cultured on t he 3D G-collagen a s a n i ncreased bi nding s trength t o t he c ollagen would i nhibit pr oliferation and m obility of t he fibroblasts.

7. 3DG-collagen induced the perinuclear localization of paxillin and focal adhesion kinase.

In our control studies, we found several dramatic changes in fibroblasts when they were cultured in an environment of 3DG-collagen. Firstly, we found that fibroblasts had increased adhesion to 3DG-collagen and i t is this increased adhesion to the collagen, through i ntegrins on t he cell surface of the fibroblast that mediate downstream s ignaling. W e f ound t his t o be s urprising but when w e f urther i nvestigated t his phenomenon, we found several abnormalities in protein expression of integrins, paxillin, and focal adhesion kinase was also altered. Integrins are the receptor for collagen and paxillin and focal adhesion kinase interact with the integrin receptor and transmit signals further downstream. We found that in fibroblasts cultured on 3DG-collagen, pa xillin and f ocal a dhesion ki nase w as l ocated t o t he p erinuclear r egion (Figure 7). We speculate th at c hanges i n the loc alization of the se pr oteins will di rectly affect s ignaling f rom th e int egrin receptor.
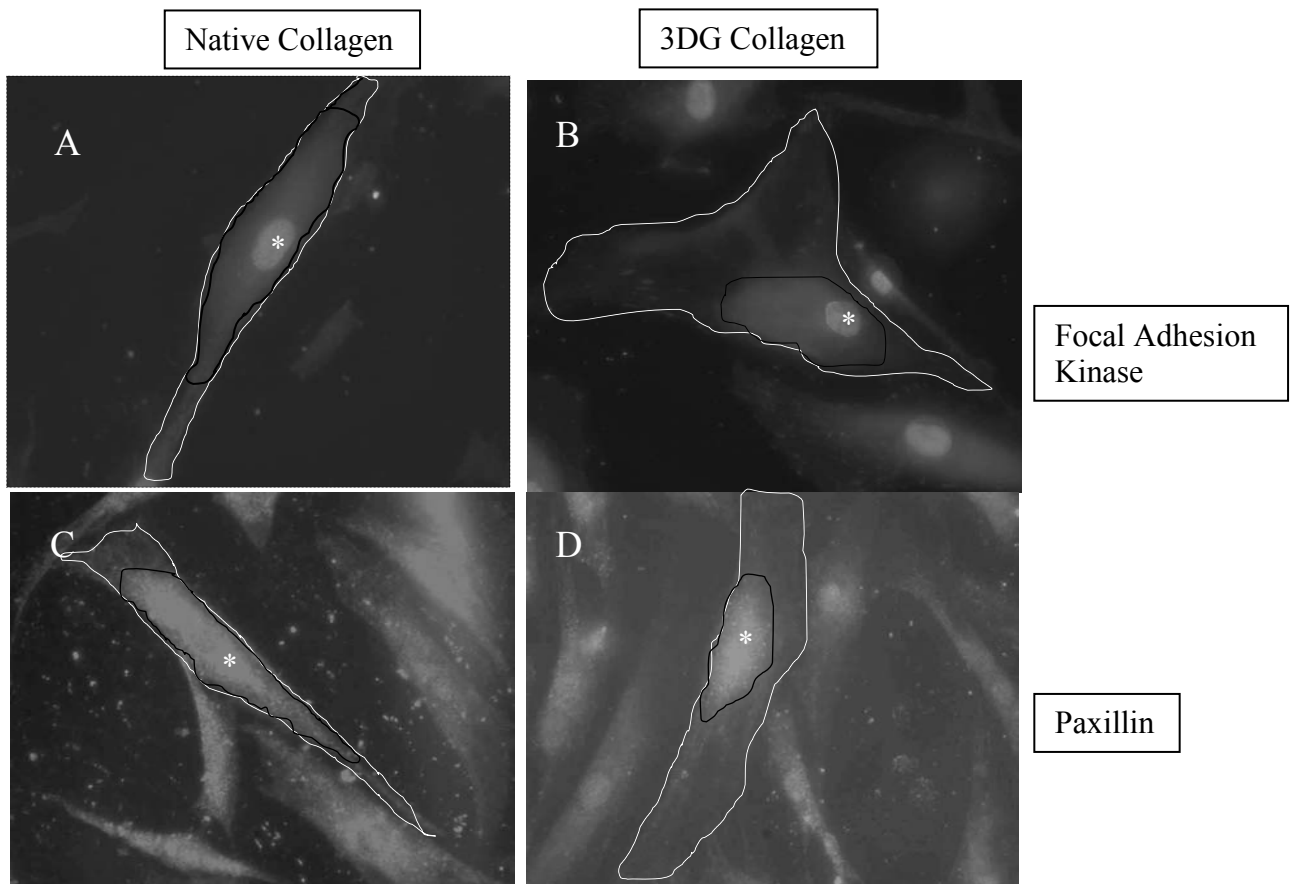

Figure 7. Localization of Paxillin and focal adhesion kinase in fibroblasts cultured on 3DG-collagen. Immunofluorescence of focal adhesion proteins, focal adhesion kinase and paxillin in subconfluent fibroblasts cultured on native collagen (A and C) and 3DG-collagen (B and D). Note the staining throughout the fibroblast for both focal adhesion kinase and paxillin when cultured on native co llagen (A an d C). H owever, when t he f ibroblasts were cu ltured o n 3 DG-collagen, t he l ocalization o f f ocal ad hesion kinase and paxillin shifted to the perinuclear region, away from the periphery of the fibroblast, closer to the nucleus (B and D). All images were t aken at 4 0X magnification o n a n ep i-fluorescence m icroscope. The n ucleus i s d enoted b y t he white s tar, the periphery of the cell has been outline with the white dotted line and the primary location of the staining has been outlined with the black line.

This finding suggested to us that right from the periphery of the cell; the signaling has been altered as focal adhesion kinase and paxillin are now no longer located immediately under the integrin receptor where they are required to transmit signals to the nucleus. We have found that ERK1/2 phosphorylation was altered and therefore, we believe that this change in protein localization has a direct effect on ERK1/2 signaling.

When we further investigated the expression of focal adhesion kinase and paxillin, we found that the expression of focal adhesion kinase was decreased by nearly 50%, whereas the expression of paxillin was not. Therefore, not only is the localization of focal adhesion kinase altered but the amount expressed is also changed (Figure 8).
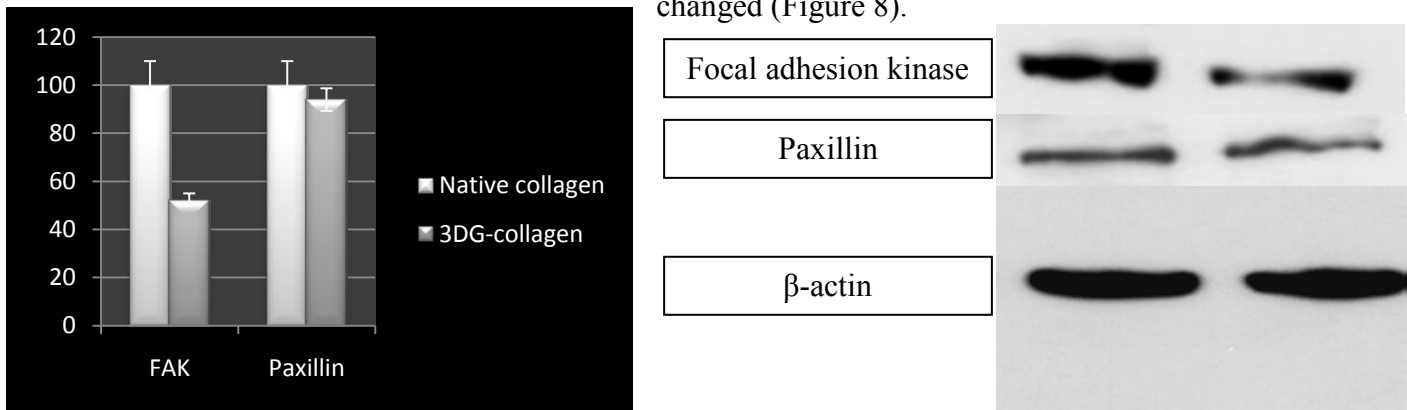

Figure 8. Decreased expression of focal adhesion kinase but not paxillin in fibroblasts cultured on 3DG-collagen. Fibroblasts were cultured on native collagen and 3DG–collagen. The PVDF was probed with an antibody directed against either focal adhesion kinase, paxillin, or β-actin. The intensity of the bands was measured by ImageJ and normalized to β-actin. It was found that focal adhesion kinase was decreased by approximately 50% in fibroblasts cultured on 3DG collagen (P < 0.002), whereas paxillin expression remained unaltered.

8. **3DG-collagen modulates the expression of integrins.**

As we had protein available for the ERK1/2 and MEK1/2 western blotting experiments, we further investigated the expression of integrins as this proved to be more cost effective with regards to tissue culture reagents and employee time. These experiments were reported in the last annual report, however we have gone on to perform more real-time assays and measure protein by Western analysis. Therefore we have already investigated some of the alterations in the expression of integrins by the 3DG-collagen, which we proposed to do in Aim 2. Integrins are receptors for collagen and other extracellular matrix proteins and they are involved in outside-to-inside signaling in the fibroblast. They directly sense the immediate environment and alter the secretion of extracellular matrix proteins. We observed changes in integrin expression and some of the integrins were increased; whereas, others were found to be decreased (Figure 9A). Comparing SSc fibroblasts cultured on native collagen to those cultured on 3DG-collagen; α1 integrin was increased by 3.5 fold (P < 0.0001), α5 integrin by 3.5 fold (P = 0.0058), αv integrin by 1.3 fold (P = 0.0009), and β3 integrin by 1.5 fold (P = 0.017); whereas α2 integrin was decreased by 50% (P = 0.0053) and β1 integrin was decreased by 30% (P < 0.0001). Concordantly, we found that this also translated into changes in protein expression (Figure 9B).
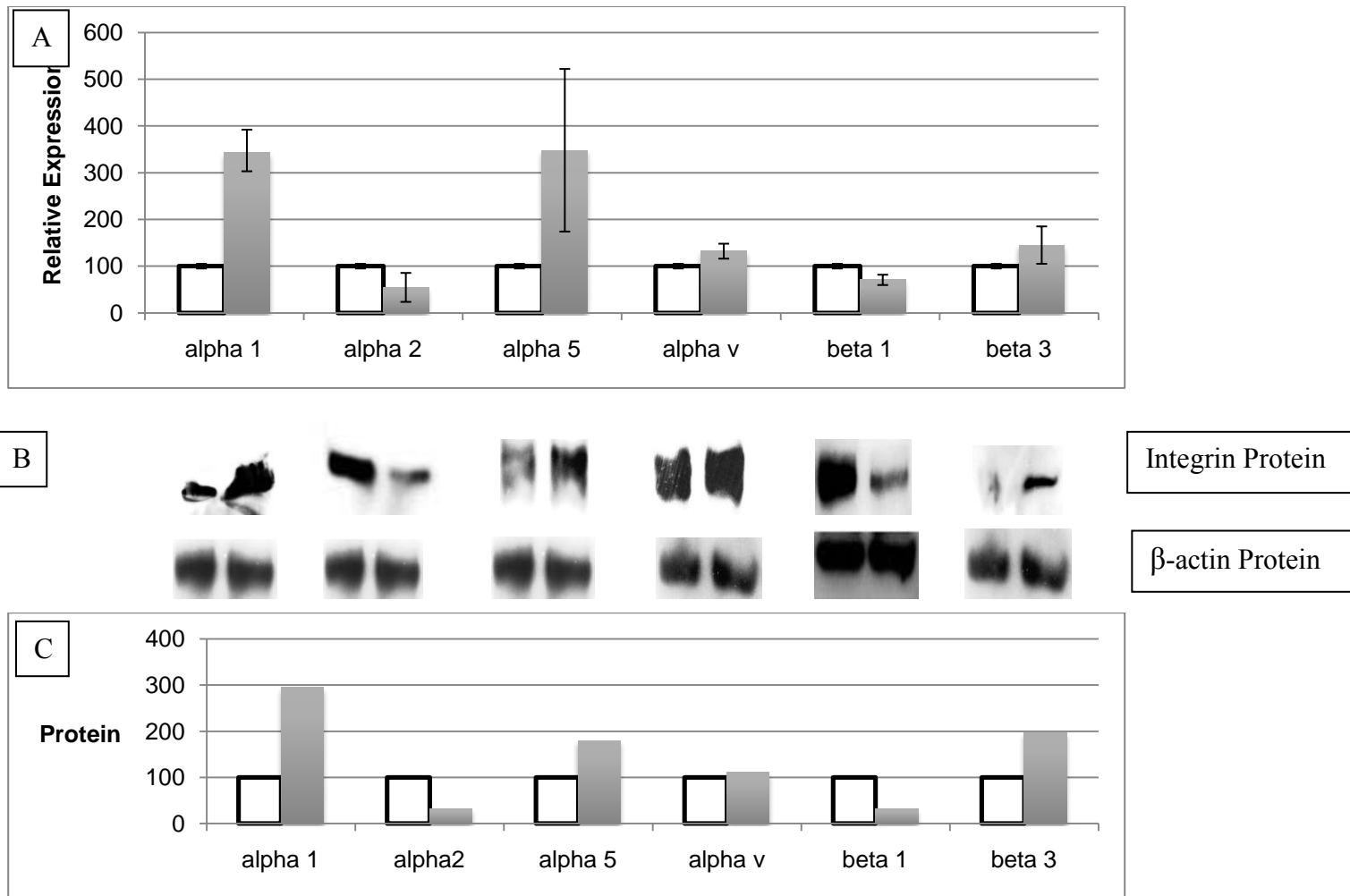
Figure 9. Alteration in expression of integrins on fibroblasts in response to 3DG-collagen. SSc fibroblasts were cultured on native collagen and 3DG-collagen, RNA was extracted and purified, and made into cDNA. The expression of integrins was measured by real-time PCR (A). Changes in integrin expression were also confirmed at the protein level (B) and graphed after corrections for β-actin as a control for protein loading (C). Comparing SSc fibroblasts cultured on native collagen to those cultured on 3DG-collagen; α1 integrin was increased by 3.5 fold (P < 0.0001), α5 integrin by 3.5 fold (P = 0.0058), αv integrin by 1.3 fold (P = 0.0009), and β3 integrin by 1.5 fold (P = 0.017); whereas α2 integrin was decreased by 50% (P = 0.0053) and β1 integrin was decreased by 30% (P < 0.0001).

We further investigated the response of fibroblasts to the 3DG-collagen and determined which integrin receptor was the most important for the fibroblast adhesion to 3DG-collagen. β1 integrin is known to heterodimerize with α1 or α2 integrin, both of which are also important for cell adhesion and proliferation of fibroblasts on collagen networks (11-13). We therefore determined the direct contribution of β1, α1, and α2 integrins in 3DG-collagen binding by preincubating the fibroblasts with monoclonal antibodies targeting these integrins; and then culturing the preincubated fibroblasts on 3DG-treated collagen and performing the jet wash assay.

Antibodies to all three integrins decreased fibroblast attachment to native collagen: after incubation with β1 integrin, 45.5% ± 2.12% of the expected number of cells remained, with α1 integrin 74% ± 1.41% remained, and with α2 integrin 53% ± 1.41% of the expected number of cells remained attached to the collagen (Figure 10A-C; P < 0.0001). With the 3DG-treated collagen, blockade of β1 integrin and α1 integrin induced a further reduction in adherence of cells to 3DG-collagen; 21% ± 2.12% of the expected cell number remained with β1 integrin and 48% ± 1.41% for α1 integrin (Figure 10D-E; P < 0.04). No difference was

observed by blocking α2 integrin (Figure 10F). These data indicates that α1β1 integrin is essential for fibroblast adhesion to 3DG-collagen.
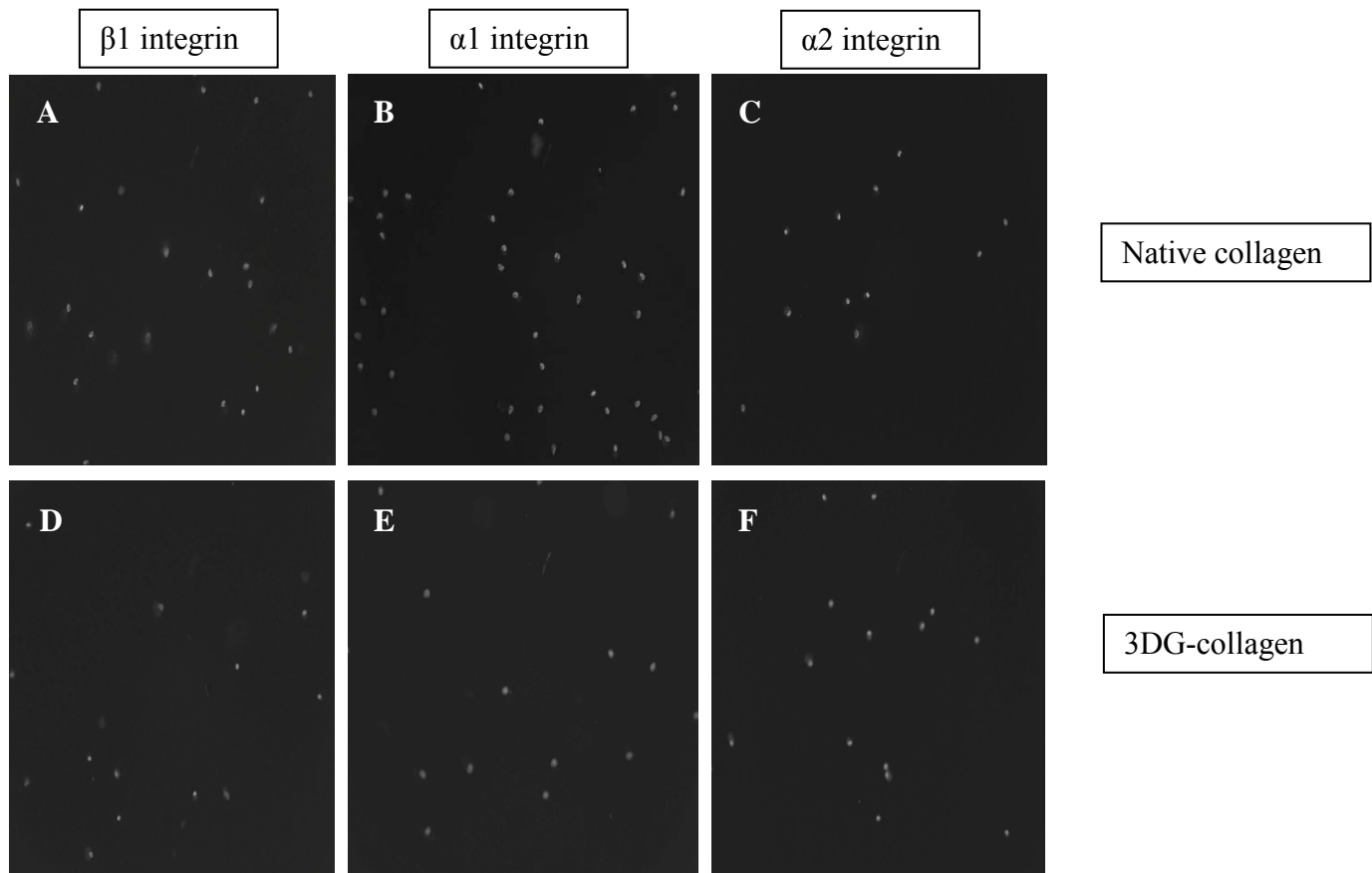

Figure 10. α1β1 integrin is involved in binding 3DG-collagen. Fibroblasts were coated with blocking antibodies for α1, α2, or β1 and allowed to attach to native or 3DG collagen. After 3 h, the fibroblasts were jet washed to remove the non-adherent cells and 10 fields of view were counted, as described above. α1β1 integrins were found to be involved in binding the 3DG-collagen, whereas α2 was not. On native collagen, blocking with β1 integrin, 45.5% $\pm$ 2.12% of the expected number of cells remained attached after the jet wash assay, 74% $\pm$ 1.41% remained with α1 integrin, and 53% $\pm$ 1.41% remained after blocking the α2 integrin (A-C; P < 0.0001). With the 3DG-treated collagen, blockade of β1 integrin and α1 integrin induced a further reduction in adherence of cells to 3DG-collagen; 21% $\pm$ 2.12% of the expected cell number remained with β1 integrin and 48% $\pm$ 1.41% for α1 integrin (D-E; P < 0.04). No difference was observed by blocking α2 integrin (F).

9. 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 (10). The characteristic marker for protein misfolding and ER stress is Growth Arrest and DNA Damage-Inducible Gene 153 (GADD153) (14). ER stress can occur due to the accumulation of membrane bound proteins in the ER, which in turn activates the transcription factor GADD153 (14). Under non-stress 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 (14). 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 over fibroblasts cultured on native collagen (Figure 11).
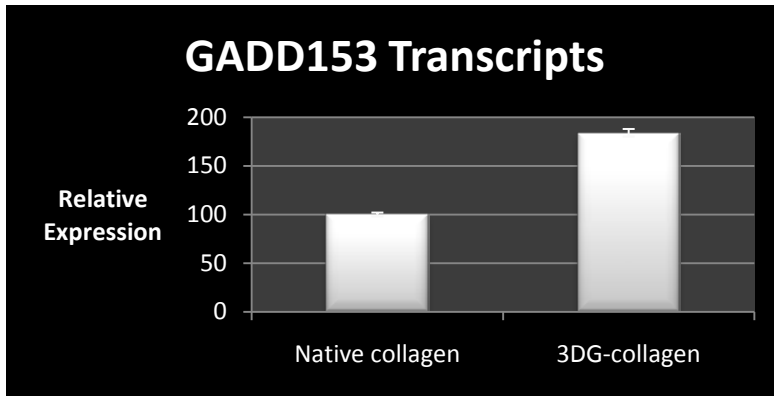
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Figure 11. GADD153 mRNA transcripts are elevated by 3DG-collagen. Fibroblasts were cultured on native or 3DG-collagen for 24 h then RNA was extracted and made to cDNA. GADD153 transcripts were measured and normalized to β-actin expression. GADD153 was found to be significantly increased in the fibroblasts cultured on 3DG-collagen P < 0.03.

Further confirming the elevation in GADD153 with 3DG-collagen, we calculated the mean fluorescent intensity (MFI) from histological analyses of fibroblast nuclei stained for GADD153. Histological analyses demonstrated that cells cultured on 3DG-collagen matrices had significant localization of GADD153 in the nucleus (66.3 MFI ± 6.641) compared to fibroblasts cultured on native collagen (43.7 MFI ± 4.307; Figure 12A & B). The differences in mean fluorescence intensity in fibroblasts cultured on 3DG-collagen vs. native collagen were significant, P < 0.0004.
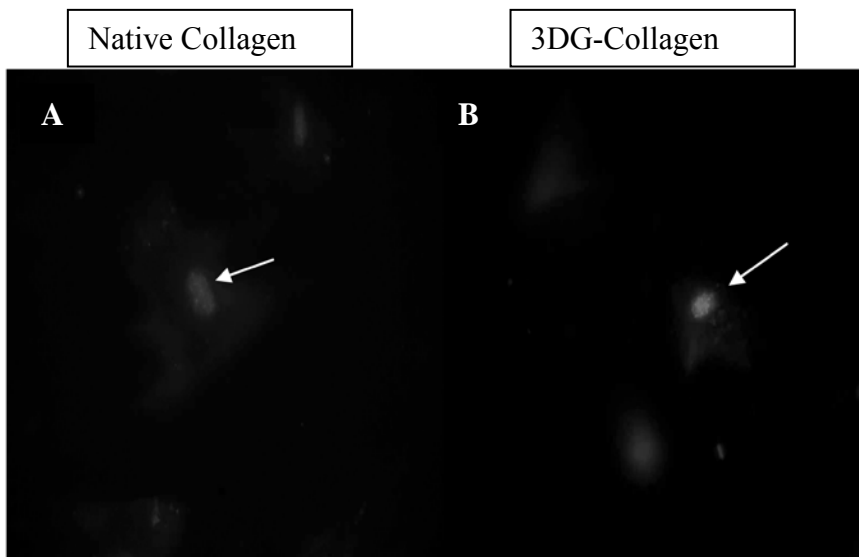

Figure 12. Localization of GADD153 in nuclei cultured on native and 3DG-collagen. Fibroblasts were cultured on native (A) or 3DG-collagen (B) in chamber slides and stained for GADD153. The intensity of the staining in the nucleus (arrow) was quantified by ImageJ. The expression of GADD153 protein was found to be significantly increased in the fibroblasts cultured on 3DG-collagen P < 0.0004.

10. Phosphorylated p38 MAP kinase is increased in the nuclei of fibroblasts cultured on 3DG-collagen.

GADD153 expression is modulated by the phosphorylation of p38 MAP kinase contributing to the increased expression of caspase-3 (see 2008 annual report). This current study has established that in vitro exposure of human fibroblasts to 3DG-collagen leads to a reduction of key focal adhesion proteins and upregulation 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 the stronger adherence of the fibroblast to the collagen matrix. Indeed, misfolding of focal adhesion proteins could cause both upregulation of GADD153, and down-regulation of ERK1/2. As the p38 MAP kinase protein is involved in modulating signals from focal adhesion kinase, we sought to determine if there were changes in the

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phosphorylation of p38 MAP kinase due to the 3DG-collagen. There was induction of p38 MAP kinase in the nucleus in fibroblasts cultured on 3DG-collagen compared to native collagen. We also found that the signal was fleeting and peaked in the nucleus at 20 min and was gone.

3DG increases phospho-p38 expression
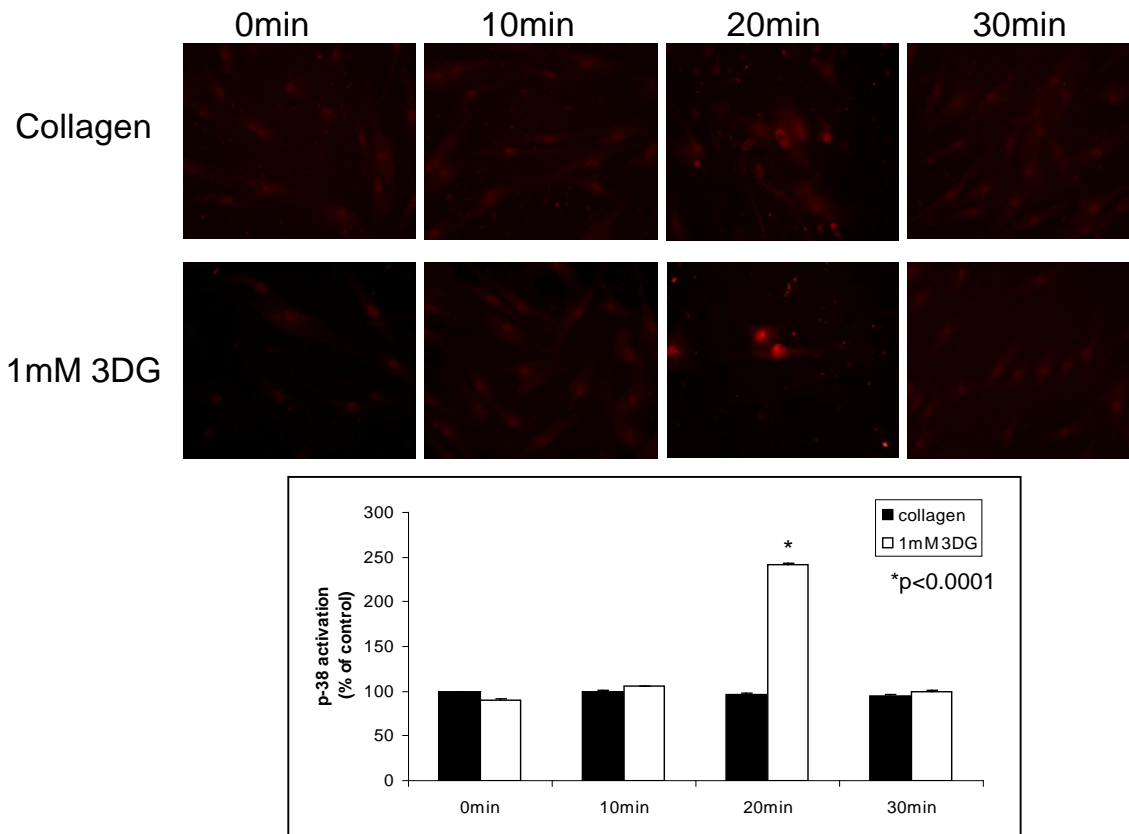
Figure 13. Increased localization of phospho-p38 MAP kinase in nuclei cultured on native and 3DG-collagen. Fibroblasts were cultured on native collagen in chamber slides and 3DG was added. Cells were fixed at specific time intervals and stained for phospho-p38. The intensity of the staining in the nucleus was quantified by ImageJ. The expression of phosphor-p38 protein was found to be significantly increased in the fibroblasts cultured on 3DG-collagen P < 0.0004 and that the signal peaked in the nucleus at 20 min.

11. Inhibition of p38 abolished GADD153 expression.

We wanted to determine if p38 had a direct effect on GADD153 expression with 3DG. Cells were cultured on native collagen and stimulated with 3DG with and without the p38 inhibitor, SB202190. GADD153 protein was measured histologically and mRNA transcripts by real-time PCR. We found that inhibiting p38; we were able to inhibit GADD153 expression (Figure 14). We are currently in the process of determining if inhibiting p38 will increase collagen expression.

Inhibition of p38 abolishes 3DG-induced GADD153 expression

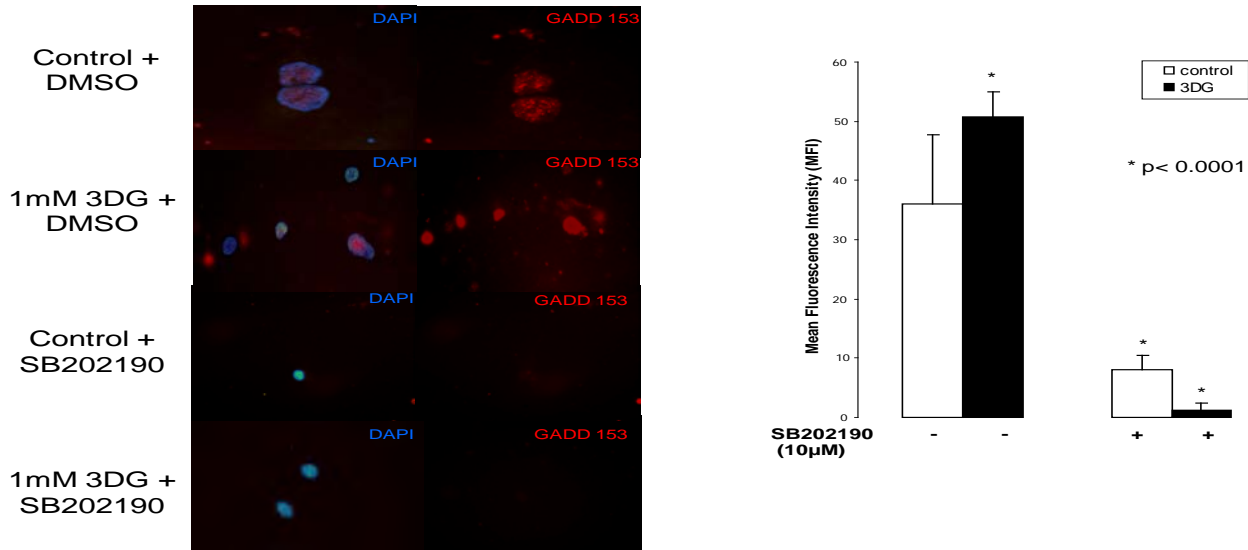**Figure 14. Inhibition of p38 MAP kinase inhibits GADD153 expression.** Fibroblasts were treated with 3DG with or without the p38 inhibitor, SB202190 and stained for GADD153. The staining in the nuclei was quantified by ImageJ and represented graphically on the right. Inhibition of p38 abolished the induced expression of GADD153 by 3DG. This was found to be statistically significant P <0.0001.

12. Smad7 protein is increased in fibroblasts cultured on 3DG-collagen.

In an effort to further elucidate how reduced TGF-β contributes to the reduced expression of COL1A1, we investigated Smad7. Smad7 regulates the response of the TGF-β signaling by interfering with TGF-β-mediated phosphorylation of other Smad family members. If Smad7 is increased, there is less phosphorylation of Smad2/3 and Smad2/3 promotes collagen expression. Phosphorylated Smad2/3 is elevated in SSc fibroblasts (15). Therefore we investigated Smad7 expression in fibroblasts cultured on 3DG-collagen (Figure 14).

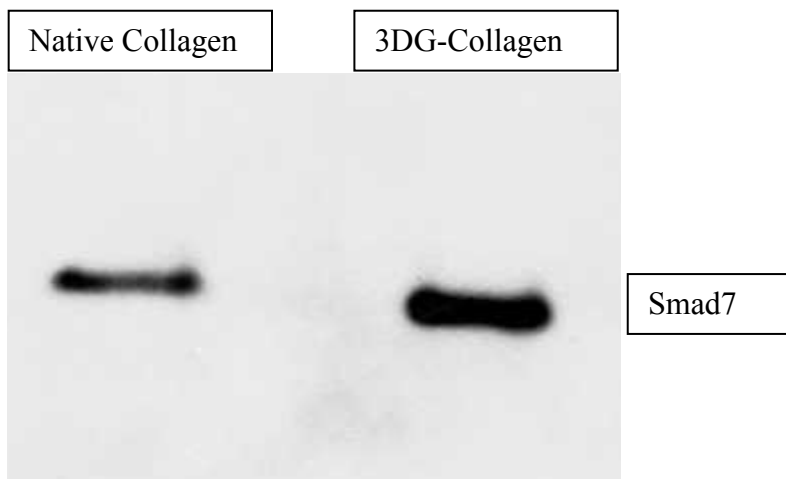**Figure 14. Smad7 protein is increased in fibroblasts cultured on 3DG-collagen.** We cultured fibroblasts on native collagen and 3DG-collagen and measured Smad7 protein in cell lysates. Smad7 was found to be increased. As we have not measure β-actin for this Western blot, we have not corrected for gel loading differences and therefore do not have statistics for this difference. This is currently being performed in the laboratory.

Key Research Accomplishments for June 2008 – May 2009.

1. 3DG-collagen decreased fibroblast ERK1/2 phosphorylation in normal fibroblasts (Figure 1)
2. 3DG-collagen decreased fibroblast ERK1/2 phosphorylation in SSc fibroblasts (Figure 1)
3. 3DG-collagen decreased fibroblast MEK1/2 phosphorylation in SSc fibroblasts but not in normal fibroblasts (Figure 2)
4. Histological analyses demonstrated reduced ERK1/2 and MEK1/2 protein in fibroblasts when cultured on 3DG collagen (Figure 3)
5. With gel shift experiments confirmed the distal Sp1/NF-1 switch element in the collagen promoter mediated collagen gene expression (Figure 4)
6. The shift in the DNA/protein band for the distal NF-1/Sp1 switch element was abolished when SSc fibroblasts are cultured on 3DG-collagen (Figure 4)
7. Sp1 mRNA transcripts were decreased by 88.7%
8. c-myc and c-fos mRNA transcripts were not altered
9. Sp1 transcription protein activity was decreased by 37% (Figure 5)
10. c-myc transcription protein activity was decreased by 30% (Figure 5)
11. c-fos transcription protein activity was decreased by 17% (Figure 5)
12. Fibroblasts were found to adhere more strongly to 3DG-collagen (Figure 6)
13. 3DG-collagen induced the perinuclear localization of paxillin and focal adhesion kinase (Figure 7)
14. Focal adhesion kinase protein was decreased when fibroblasts were cultured on 3DG-collagen, however paxillin was not (Figure 8)
15. 3DG-collagen induced mRNA and protein expression of α1 integrin (Figure 9)
16. 3DG-collagen induced mRNA and protein expression of α5 integrin (Figure 9)
17. 3DG-collagen induced mRNA and protein expression of αv integrin (Figure 9)
18. 3DG-collagen induced mRNA and protein expression of β3 integrin (Figure 9)
19. 3DG-collagen decreased mRNA and protein expression of α2 integrin (Figure 9)
20. 3DG-collagen decreased mRNA and protein expression of β1 integrin (Figure 9)
21. α1β1 integrin is involved in binding 3DG-collagen (Figure 10)
22. 3DG-collagen induced the expression of GADD153 mRNA transcripts and protein (Figure 11 & 12)
23. Phosphor-p38 protein is elevated in the nuclei of fibroblasts cultured on 3DG-collagen (Figure 13)
24. Inhibition of p38 abolished the induction of GADD153 by 3DG (Figure 14)
25. Smad7 is increased in fibroblasts cultured on 3DG-collagen (Figure 15)

We have completed Aim 1 of the grant application.

Reportable Outcomes

Abstract Presentations at Meetings

*Artlett CM, Sassi-Gaha S, Loughlin DT. Decreased Expression of Pertinent Extracellular Matrix Molecules in Systemic Sclerosis Utilizing a Glucose Metabolite that Modulates p44/p42 MAP Kinase pathway. American College of Rheumatology, October 2008.

Loughlin DT, Artlett CM. 3-Deoxyglucosone Modifies Fibroblast Focal Adhesions and Induces GADD153 Leading to Altered Wound Healing. Discovery Day 2008.

Sassi-Gaha S, Artlett CM. 3-Deoxyglucosone Alters Integrin Expression and Collagen Specific Transcription Factors in Scleroderma Fibroblasts. Discovery Day 2008.

* This was a podium presentation at the American College of Rheumatology Conference in San Francisco, October 2008 in the Scleroderma Concurrent session. Six abstracts were presented in this concurrent session, selected from 137 accepted abstracts for scleroderma and out of a total of 2076 accepted abstracts for the entire meeting.

Lecture

Loughlin DT, Artlett CM. Role of 3-deoxyglucosone in impaired wound healing: building a mechanism. Presented to Microbiology and Immunology, Drexel University College of Medicine, April 2009.

Conclusion

We have started to elucidate the role that 3DG has in cellular signaling in determining the signaling cues that down regulates the expression of collagen genes. We believe that this research will yield important clues and ultimately will point to a suitable therapy for SSc, which to date is an incurable disease. We have found that we are modulating signaling right from the integrin receptor (Figure 15). We found that 3DG-collagen altered the expression of various integrins and that many of these integrins are important in SSc.
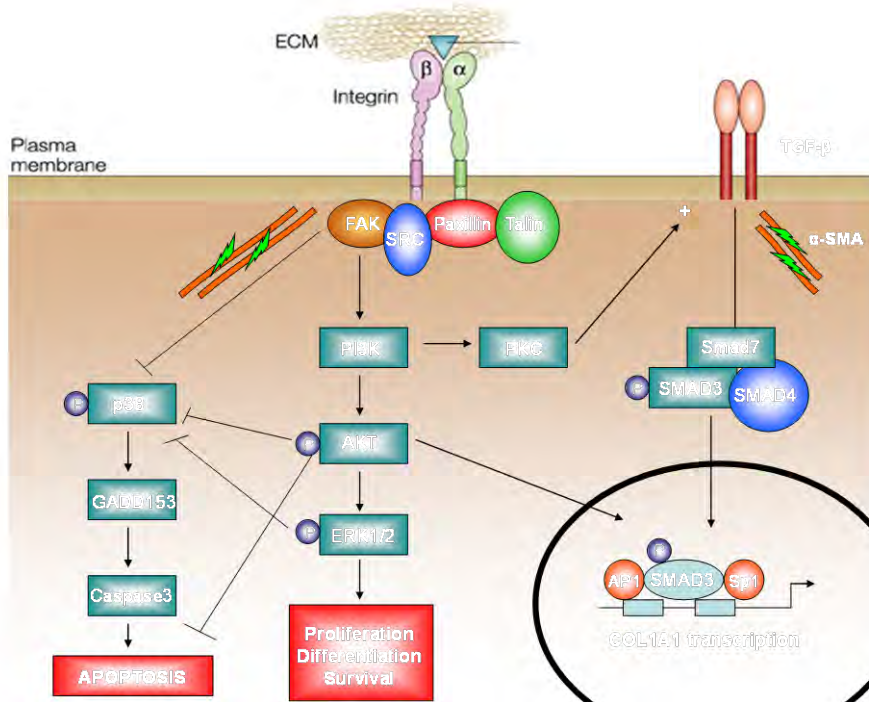
Figure 15. Schematic of the Signaling Alterations in Fibroblasts cultured on 3DG-collagen.

The $\alpha1\beta1$ integrin complex was important for binding 3DG-collagen. From the changes in integrin signaling, we found that focal adhesion kinase was decreased and focal adhesion kinase and paxillin become located to the perinuclear region away from the integrin receptors. This appears to induce a cascade of signaling that not only affected the MEK1/2-ERK1/2 pathway but also affected the p38 pathway leading to GADD153 expression.

We found that Smad7 was increased in fibroblasts cultured on 3DG-collagen. Smad7 is an important regulator in mediating TGF-β signals and the balance between Smad7 and Smad2/3 and the expression of other transcription factors determines whether collagen is expressed. Confirming that Smad7 is increased directly suggests that Samd2/3 signaling is interrupted resulting in the observed decrease in collagen expression.

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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.

Abstracts have been included.

Decreased Expression of Pertinent Extracellular Matrix Molecules in Systemic Sclerosis Utilizing a Glucose Metabolite that Modulates p44/p42 MAP Kinase pathway.

Carol M. Artlett, Sihem Sassi-Gaha, Danielle T Loughlin. Drexel University College of Medicine.

Purpose: Scleroderma (SSc) is a fibrotic disease of unknown origin. What is apparent is the uncontrolled fibrosis in the dermis and internal organs that affects morbidity and leads to mortality in these patients. Understanding the mechanisms whereby fibroblasts interact with the extracellular matrix (ECM), are central to the understanding of fibrosis in SSc. TGF-beta signals through the p44/p42 MAP kinase pathway inducing the fibrogenic pathology observed in SSc. Glucose metabolites have been implicated in numerous pathologies but more importantly they have been implicated in collagen cross-linking. Therefore, we undertook analyses that investigated the role of one of these metabolites, Dyn18, in the modulation of ECM gene expression in SSc dermal cell lines.

Methods: Primary dermal cell lines from the active lesions from patients with diffuse SSc were obtained from Carol Feghali-Bostwick at Pittsburgh University and re-established in culture. Fibroblasts were treated with Dyn18 and cultured 24 h before harvesting for RNA. cDNA transcripts of COL1A1, COL3A1, elastin, fibrillin-1, CTGF, and TGF-beta genes were measured in SSc and compared to untreated SSc fibroblast cell lines. We also investigated the phosphorylation of p44/p42 MAPK in SSc fibroblasts treated with Dyn18 and compared it to untreated fibroblasts.

Results: With only 24 h incubation, we found that the glucose metabolite, Dyn18, significantly decreased the expression of ECM molecules in the dermal fibroblast lines from the SSc patients: COL1A1 decreased by 24%, COL3A1 by 23%, TGF-beta by 70%, fibrillin-1 by 53%, elastin by 6%, and CTGF by 35%. We also found that we reduced the phosphorylation of p44/p42 MAPK by 40%.

Conclusions: We demonstrate for the first time, that a glucose metabolite can be utilized to decrease the expression of pertinent ECM proteins that are over expressed in SSc dermal fibroblasts. We also found that we decreased the phosphorylation of p44/p42 MAP kinase confirming that this pathway is integrally involved in the over expression of ECM proteins in SSc. Taken together this data is provocative and suggests a newly discovered therapeutic that may be effective in controlling fibrosis in patients with SSc.

3-Deoxyglucosone Alters Integrin Expression and Collagen Specific Transcription Factors in Scleroderma Fibroblasts
Sihem Sassi-Gaha and Carol M. Artlett

Systemic sclerosis (SSc) is a fibrotic disease of unknown origin. We previously demonstrated that collagen was decreased by SSc dermal fibroblasts with the advance glycation end-product, 3-deoxyglucosone (3DG) and this led us to speculate that this was mediated through collagen receptors on the cell surface. Integrins bind collagen and sense the environment external to the cell and allow the cells to respond to that environment. Therefore, we investigated the expression of integrins on SSc fibroblasts isolated from the fibrotic active lesions cultured on collagen that had been modified by 3DG. 3DG decreased the expression of integrin alpha2 and beta1, but induced the expression of alpha1, alpha5, alphav, and beta3, while decreasing type 1 collagen and TGF-beta. When we investigated the transcription factors that are known the modulate collagen, we found c-myc, c-fos and Sp1 protein were decreased. We also found that elastin, fibrillin and alpha-smooth muscle actin mRNA transcripts were depressed, suggesting that the modification of lysine and arginine by 3DG is important and can affect the expression of multiple extracellular matrix proteins. Indeed, culturing fibroblasts on non-modified collagen matrices and supplementing with 3DG-modified arginine depressed collagen expression. Further studies are underway to understand the effect of 3DG on the fibroblast and to determine if this can be utilized as a potential therapeutic for SSc.

3-Deoxyglucosone Modifies Fibroblast Focal Adhesions and Induces GADD153 Leading to Altered Wound Healing
Danielle T Loughlin and Carol M. Artlett

The interaction of fibroblasts with the extracellular matrix (ECM) is critical for wound healing and for the functional integrity of the tissue. Advanced end glycation products (AGEs) occur as a result of fructose and glucose metabolism resulting in the glycation of long lived proteins such as collagens. This alteration leads to abnormal pathophysiology of the ECM which may complicate chronic wounds. One of the precursors to AGE is 3-deoxyglucosone (3DG). 3DG has been found to be elevated in patients with diabetes and accumulates on collagen with increasing chronological age. Since the process of wound repair is dependent on fibroblast migration, proliferation, and expression of ECM proteins at the wound site, we examined the role of 3DG- modified collagens and the subsequent response of fibroblasts to this modification. We demonstrate that fibroblasts adhere more strongly to 3DG-modified collagen, express less collagen, and are unable to migrate efficiently into the wound site, when compared to unmodified collagen. This suggested an impaired organization of the actin cytoskeleton. Focal adhesion kinase and paxillin are important proteins that make up focal adhesions (FA), which are involved in cell migration. We further show that cells treated with 3DG exhibit unorganized actin cytoskeleton and different localization of key FA proteins. Additionally, these cells express higher levels of the misfolded indicator protein GADD153. This data suggests that fibroblast/matrix interactions alter as AGEs accumulate and affect focal adhesion formation. These findings also suggest that 3DG may be an 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.

DREXEL UNIVERSITY
COLLEGE OF MEDICINE

Office of Research Compliance
APPROVAL NOTICE (EXEMPT)

TO: Carol M. Artlett , PhD

Total Pediatrics / Immunology

Mailstop: QL

FROM:

Michael P O'Connor

Michael P. O'Connor , M.D., Ph.D , Vice-Chair
Institutional Review Board (IRB #1)
Drexel University College of Medicine
1601 Cherry Street, Philadelphia, PA 19102
Tel: 215-255-7866 Fax: 215-255-7874

SUBJECT: EXEMPT APPROVAL

TITLE: Modulation of Fibrosis in Scleroderma by 3-Deoxyglucosone

SPONSOR: Department of Defense

PROJECT No: 71434, PROTOCOL No: 16793 , ACTION No: 47834 Type: Periodic Report Period: 2 Seq: 1 , DETAIL No: 241783

CURRENT APPROVAL PERIOD: 01/14/2008, EXPIRES: 01/13/2009

RE: 01/14/2008 - Approved Exempt Renewal. Approval Includes: 16 Cell Lines Already Established and Obtained from Pittsburgh University to Complete the Study.

Date: 1/18/2008

On behalf of the Committee, I am pleased to inform you that the subject protocol has been reviewed and approved as **EXEMPT research** (45 CFR 46, 101(b)(7)) for the period indicated above. We operate under many Government requirements. As a result, this approval is granted with the following understandings:

1. If this is a sponsored project, then the study may not be activated until the Clinical Research Group has received BOTH a fully executed sponsored agreement AND appropriate letter(s) of indemnification by the sponsor. If this is not a sponsored study (designated "internal"), the costs of the project must be identified and a cost center designated. Please call 215-762-3453 if you have any questions regarding these procedures.
2. You must advise the IRB of the activation date. Use the attached form for this purpose.
3. Protected Health Information (PHI) cannot be collected without a Waiver of Authorization per HIPAA regulations.
4. Any change to the protocol must be submitted in writing and approved by the IRB in advance.
5. Any adverse reaction must be reported to the IRB as soon as it occurs.
6. Should the IRB decide to monitor your project directly, please cooperate fully. Failure to do so may result in withdrawal of this approval and notification to the sponsor and/or Federal agencies. Specific information regarding monitoring appears in the book: "Guidelines for Biomedical and Behavioral Research Involving Human Subjects", obtainable through this office or vi the website http://research.drexel.edu.
7. Whether or not this protocol is activated, the IRB will conduct a Continuing Review at least annually. Should you fail to respond to this Federally-required progress report, the project may become ineligible for re-approval and the IRB may choose not to consider other projects for approval.
8. A final progress report must be submitted to the IRB in format similar to that of a periodic report.

The IRB welcomes your research project into the list of approved protocols. Your compliance with the above conditions will help to protect the continuation of all research activity at the University. With your project and others like it, we look forward to additions to knowledge of human health and benefits to science, our patients, and society.

cc: Dept Chair, Tenet, and Drexel

In the tradition of Woman's Medical College of Pennsylvania and Hahnemann Medical College®

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DREXEL UNIVERSITY
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Office of Regulatory Research Compliance

MEMORANDUM

TO: Carol M. Artlett
            Total pediatrics / Immunology
            Mailstop: QL

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

Please be informed that Exempt Research Protocols without HIPAA are exempt from continuing review and an approval for continuation is no longer required. However, it is the Principal Investigator's responsibility to notify the Office of Regulatory Research Compliance as soon as the research has been completed. Use the Periodic Report form, which may be obtained from www.research.drexel.edu and submit a final report.

If you require additional information, please contact the Office of Regulatory Research Compliance at 215-255-7857.

Thank you.

Attach: Investigator's Responsibilities

INVESTIGATOR'S RESPONSIBILITIES

Drexel University College of Medicine operates in compliance with all applicable laws and regulations and good clinical practice. Drexel University relies upon Drexel University College of Medicine's IRBs for the review and approval of all protocols. Investigators are responsible for the implementation of protocols as approved by the IRB. Formal guidance to investigators, standard operating procedures of IRB and forms to be used are posted on the Office of Regulatory Research Compliance website www.research.drexel.edu. Additional educational information can be obtained at www.irbforum.research.drexel.edu.

Consent Forms

Only approved and stamped consent forms must be used to enroll subjects. It is against regulations to use expired consent forms. Informed consent will be sought and documented in accordance with, and to the extent required by Federal regulations. When appropriate, there must be adequate provisions to protect the privacy of subjects and to maintain the confidentiality of data. The PI will insure that informed consent is obtained from each subject prior to the start of his or her participation in a clinical or behavioral research study. Only an IRB approved and stamped consent form is to be used for consenting subjects.

Please note that the consent document is a written summary of the information that should be provided to the subject. Many investigators use the consent document as a guide for the verbal explanation of the study. The subject's signature provides documentation of agreement to participate in a study, but is only one part of the consent process. The entire informed consent process involves giving a subject adequate information concerning the study, providing adequate opportunity for the subject to consider all options, responding to the subject's questions, ensuring that the subject has comprehended this information, obtaining the subject's voluntary agreement to participate and, continuing to provide information as the subject or situation requires. To be effective, the process should provide ample opportunity for the investigator and the subject to exchange information and ask questions. Federal regulations require a copy of the consent document be given to the prospective subject. Verbal consent does not satisfy the requirement for signed consent document.

Continuing Review and Re-approval

It is the investigator's responsibility to apply for continuing review ongoing research annually or at a frequency IRB has approved. The ORRC will send sixty (60) and thirty (30 days) reminders to Investigators in advance of the expiration date. Forms for continuing review called Periodic Report Form can be obtained from www.research.drexel.edu. Forms must be submitted to the ORRC at least 21 days ahead of the expiration date.

Continuing review of research projects to be remained as active for follow up and data analysis also requires IRB approval. For such requests, complete the Periodic Report Form indicating that the study is open for follow up or data analysis. There is no need to submit a blank consent form for re-approval for those purposes.

Exempt projects without HIPAA. For such projects, a Periodic Report for continuing review is not required.

Completion of Study

It is the Principal Investigator's responsibility to notify the Office of Regulatory Research Compliance as soon as the research has been completed. If the study is going to be closed at the time of protocol expiration date, use the Periodic Report form, which may be obtained from www.research.drexel.edu and submit a final report.

Adverse Reactions

If any adverse events or unanticipated problems that pose risk to subjects or others as a result of this study, you are required to report such risks to the ORRC using the Adverse Event Report Form. All serious and expected adverse events that are probably or definitely related to the project must be reported within five days. Serious

and unexpected adverse events that are probably or definitely related must be reported within 24 hours. Adverse events reporting forms can be downloaded from www.research.drexel.edu and completed forms can be submitted via e-mail. For serious problems, approval may be suspended pending further IRB review.

Changes and Amendments
All changes to the protocol whether minor or significant must be approved by the IRB prior to implementation. It is the responsibility of the Principal Investigator to keep the IRB informed of any changes in the protocol, its participating investigators, procedures, recruitment, consent forms, FDA status of the investigational agents or devices and conflict of interest. Any changes to this protocol must be presented using the form entitled **AMENDMENTS, CHANGES AND PROJECT TERMINATION**. This form can be downloaded from the Office of Research website www.research.drexel.edu.

Number of Subjects to be Enrolled
Please pay particular attention to total number of subjects permitted to be enrolled in the study. This number is defined as the number of participants who sign the informed consent document. If this number is reached, enrollment must cease. Enrollment of participants beyond the initial approved number is considered non-compliance with terms of the project approval. Investigators should not enroll more subjects than the number specified in the study protocol that is currently approved by the IRB. The IRB recognizes that there are times when enrollment in a study must be increased. Investigators may submit an amendment to the protocol using the "Amendment Form" posted on www.research.drexel.edu. Enrollment shall not continue above the original sample size until the IRB has approved the increase in number of subjects.

Record Keeping
Organized record keeping is crucial to the research process. The clock starts ticking the day the protocol was approved whether research subjects are enrolled or not. All correspondence between investigator and IRB should be kept together in a binder or folder. All signed, informed consent forms (ICFs) should be kept in an organized fashion as well. Any research records that contain information along with subject identifiers should be kept in a secure location. The Office of Regulatory Research Compliance and Regulatory agencies may reserve the right to review research records. These reviews are more easily conducted when research records are kept in a systematic and organized manner.
The following tips and the information on our website www.research.erexel.edu may assist you in keeping your research records both orderly and confidential.

- Keep research records in a secure, locked location.
- "Keys" to coded information should be kept private and locked securely away as well.
- File all IRB correspondence routinely in a binder or folder and in chronological order.
- Keep a clean copy of the current version of the Protocol.
- Keep a current, clean, IRB-stamped copy of your Informed Consent Form.
- Keep all informed consent documents signed by all subjects.
- Keep track of all study changes/amendments.
- Keep track of the study expiration date.