Akt Blockade Downregulates Collagen and Upregulates MMP1 in Human Dermal Fibroblasts

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Acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (Akt) is a serine/threonine kinase that plays important roles in survival, cell-cycle progression, and cell proliferation, and has recently been implicated in collagen regulation. The aim of this study was to determine the role of Akt in collagen deposition by normal dermal fibroblasts, and to determine the sensitivity of cultured systemic sclerosis (SSc) fibroblasts to Akt inhibition. We show that blockade of Akt using pharmacological inhibitors, small interfering RNA (siRNA), and a dominant-negative Akt mutant led to inhibition of the basal type I collagen production. Furthermore, inhibition of Akt upregulated basal matrix metalloproteinase 1 (MMP1) production and reversed the inhibitory effect of transforming growth factor-β (TGF-β) on MMP1 gene expression. In addition, SSc fibroblasts were more sensitive to Akt inhibition, with respect to collagen and MMP1 production. These findings suggest that in human dermal fibroblasts, Akt has dual profibrotic effects, increasing collagen synthesis and decreasing its degradation via downregulation of MMP1. Akt could directly contribute to elevated collagen in SSc fibroblasts and it may represent an attractive target for therapy of SSc fibrosis.

Journal of Investigative Dermatology (2008) **128,** 1906–1914; doi:10.1038/jid.2008.39; published online 6 March 2008

INTRODUCTION

Akt is a key enzyme in signal transduction pathways involved in cell survival, glycogen synthesis, cell-cycle progression, angiogenesis, and migration (Kim and Chung, 2002; Hennessy *et al.*, 2005). The three Akt isoforms (Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ) are ubiquitously expressed and to some extent have overlapping functions. Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K) by growth factors, stimulators of G-protein-coupled receptors, and integrins (Kim and Chung, 2002). Following PI3K activation, Akt is recruited at the plasma membrane via binding of phosphoinositol lipids to the Akt pleckstrin homology domain. At the membrane, Akt is activated by phosphorylation on two different residues, Thr 378 by the phosphoinositol-dependent kinase–phosphoinositide-dependent kinase 11, and Ser 473 by the mTOR/rictor complex (Sarbassov *et al.*, 2005). Increasing evidence suggests that Akt may play a role in fibrosis (Chen *et al.*, 2005; Krepinsky *et al.*, 2005; Vittal *et al.*, 2005; Wu *et al.*, 2007). Fibrosis is a complex process that involves overproduction of collagen by activated fibroblasts.

Collagen type I, the main constituent of the extracellular matrix, is a triple helix composed of two α-1 and one α-2 chains, the products of *COL1A1* and *COL1A2* genes (Cutroneo, 2003). The expression of these genes is regulated at multiple levels by numerous cytokines and transcription factors. Increased production of collagen and other extracellular matrix components is a characteristic feature of systemic sclerosis (SSc) (Varga and Abraham, 2007). Despite intense efforts, the signaling pathways that regulate collagen synthesis in SSc fibroblasts are not fully defined (Jimenez and Derk, 2004). A unique feature of SSc fibroblasts is the maintenance of the activated phenotype for several passages in culture. This makes cultured SSc fibroblasts a valuable *in vitro* model to study SSc. The excessive collagen accumulation in SSc fibroblasts is a result of increased synthesis as well as decreased degradation rate. Published data show that MMP1, an enzyme that is degrading fibrilar collagen, is downregulated in SSC cells (Takeda *et al.*, 1994) and in response to transforming growth factor-β (TGF-β).

TGF-β is one of the most potent profibrotic cytokines and the major factor involved in fibroblast activation during chronic fibrosis. In most cell types, TGF-β regulates collagen via the canonical Sma- and Mad-related protein (Smad) pathway by binding to and activating specific type I and type

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Abbreviations: AdTGF-βRI, adenovirus expressing TGF-β type I receptor; Akt, acutely transforming retrovirus AKT8 in rodent T cell lymphoma; CAT, chloramphenicol acetyltransferase; MMP1, matrix metalloproteinase 1; PI3K, phosphatidylinositol 3-kinase; PK, protein kinase; qRT–PCR, quantitative real-time–PCR; RT–PCR, reverse transcriptase–PCR; siRNA, small interfering RNA; α-SMA, α-smooth-muscle actin; Smad, Sma- and Mad-related protein; SSc, systemic sclerosis; TGF-β, transforming growth factor-β; TGF-βRI, TGF-β type I receptor

Received 4 September 2007; revised 8 January 2008; accepted 16 January 2008; published online 6 March 2008

II serine/threonine kinase receptors. This results in phosphorylation and activation of Smad3 at the C-terminal SSXS motif, followed by its nuclear translocation (Chen *et al.*, 1999, 2000; Flanders, 2004). There is evidence that TGF-β-dependent signaling plays a key role in SSc fibrosis. Elevated levels of TGF-β receptors (Kawakami *et al.*, 1998; Kubo *et al.*, 2002; Yamane *et al.*, 2002; Pannu *et al.*, 2004) and increased phosphorylation and nuclear localization of Smad2 and 3 (Mori *et al.*, 2003) were reported in SSc fibroblasts, suggesting a role for the canonical pathway in SSc fibrosis. However, blockade of the canonical Smad pathway using pharmacological inhibitors of TGF-β type I receptor (TGF-βRI)/ALK5 kinase failed to completely normalize the SSc phenotype (Chen *et al.*, 2006). In addition to Smads, TGF-β receptors can induce parallel signaling pathways, which can either regulate Smad signaling or lead to Smad-independent TGF-β responses (Derynck and Zhang, 2003). Increasing evidence suggests that Smad3-independent downstream effectors could also be involved in TGF-β-mediated fibrosis (Wang *et al.*, 2005; Cho and Yoo, 2007; Pannu *et al.*, 2007). TGF-β increases Akt phosphorylation in dermal fibroblasts and Akt is constitutively phosphorylated in SSc biopsies (Jun *et al.*, 2005), also suggesting a possible role for Akt activation in collagen synthesis in SSc fibroblasts.

The aim of our study was to establish the role of Akt in type I collagen production in human dermal fibroblasts and its contribution to SSc fibrosis. Our data demonstrate that Akt is a positive regulator of collagen gene expression and a negative regulator of MMP1 synthesis in dermal fibroblasts. Furthermore, our data show that SSc fibroblasts are more sensitive to Akt inhibition.

RESULTS

Akt inhibition significantly decreases basal collagen levels in human dermal fibroblasts

To test whether Akt regulates collagen synthesis, dermal fibroblasts were treated with increasing doses of two different pharmacological Akt inhibitors (VIII and II) and COL1A1 and COL1A2 mRNA levels were measured by quantitative real-time reverse transcriptase–PCR (RT–PCR) (qRT–PCR). Treatment with Akt inhibitor VIII resulted in dose-dependent inhibition of both chains of type I collagen (Figure 1a), as well as collagen I protein levels (Figure 1b). Similar results were obtained with Akt inhibitor II (Figure 1c). A time course of collagen downregulation in the presence of Akt inhibitor VIII is shown in Figure 1d. COL1A1mRNA levels were more rapidly affected, with significant decrease at 12 hours, but both chains showed similar decrease at 48 hours (~70%).

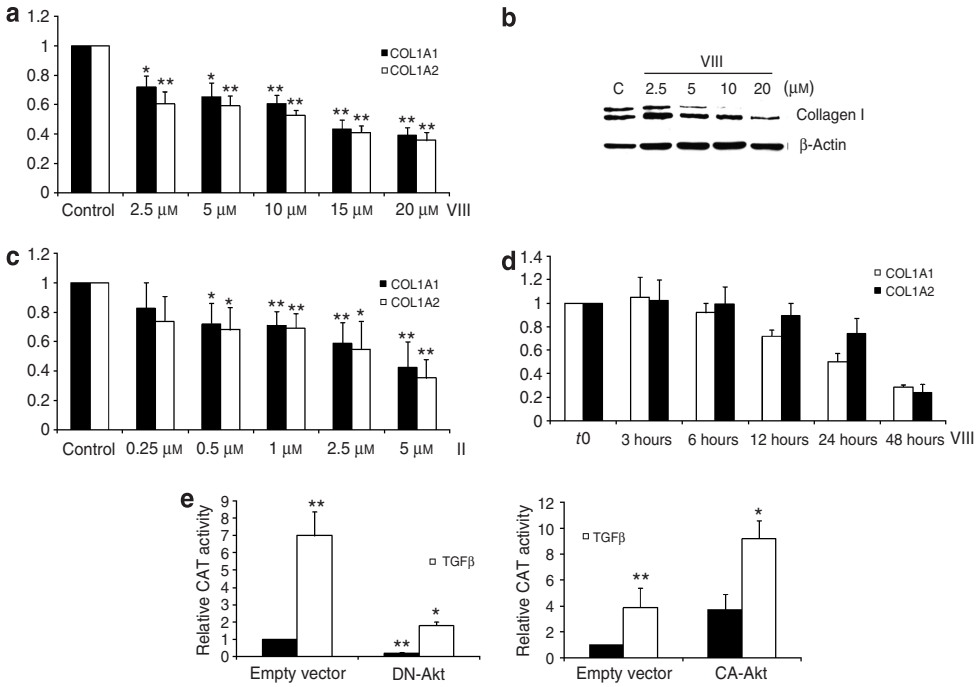

Figure 1. Akt inhibition significantly decreases basal collagen levels in human dermal fibroblasts. (**a**) Normal dermal fibroblasts were incubated in the presence of the indicated concentrations of inhibitor VIII or DMSO for 48 hours and mRNA levels of COL1A1 and COL1A2 were quantified by qRT–PCR. (**b**) Normal dermal fibroblasts were incubated in the presence of the indicated concentrations of inhibitor VIII or DMSO for 48 hours and collagen protein levels were analyzed by western blot; β-actin was used as control. (**c**) Normal dermal fibroblasts were incubated for 48 hours with the indicated concentrations of inhibitor II or DMSO and mRNA levels were quantified by qRT–PCR. (**d**) Normal dermal fibroblasts were treated with 20 μM of inhibitor VIII; cells were collected at the indicated time points and mRNA levels of COL1A1 and COL1A2 were quantified by qRT–PCR. For all the experiments, mRNA values were normalized to control (1) and the means ± SEM of at least three independent experiments are shown. *$P<0.05$; **$P<0.01$ *versus* control, DMSO treated cells. (**e**) Effect of Akt on the human −772*COL1A2/CAT* promoter. Transient transfections of foreskin fibroblasts with either empty vector (pcDNA3.0-CMV), dominant-negative Akt, or constitutively active Akt expression vectors were performed as described. Transfection efficiency was normalized using pSV-β-galactosidase control vector. Graphs represent quantitation of at least three independent experiments ± SEM, with values normalized to the activity of the promoter co-transfected with the control, empty vector, which was arbitrarily set at 1.

To further investigate Akt-dependent collagen regulation, we examined the effects of Akt blockade on collagen gene transcription. For these experiments, we co-transfected the −772*COL1A2*/chloramphenicol acetyltransferase (CAT) collagen promoter construct with plasmids overexpressing either a constitutively active or a dominant-negative form of Akt (Bellacosa *et al*., 1998). Transient overexpression of constitutively active Akt increased COL1A2 promoter activity by 3.6-fold in a manner similar to TGFβ, whereas overexpression of dominant-negative Akt reduced it by 4- and 4.6-fold in the presence of TGFβ, suggesting that the effect of Akt on collagen is partially promoter mediated. However, at this point we cannot exclude additional mechanisms, including an increase in collagen mRNA stability, as previously reported by Ricupero *et al*. (2001) for COL1A1. Taken together, these results suggest that in human dermal fibroblasts, Akt is involved in the regulation of basal collagen synthesis.

Akt inhibition has no effect on TGF-β-stimulated collagen levels

There is evidence that Akt is activated downstream of TGF-β in different experimental models, including dermal fibroblasts (Bakin *et al*., 2000; Kim *et al*., 2002; Runyan *et al*., 2004; Jun *et al*., 2005). We observed that stimulation of cells with TGF-β induced a prolonged increase in the levels of phospho-Akt, which was evident within 5 minutes after agonist treatment, reached a maximum at 2 hours, and remained increased at 24 hours (Figure 2a). Pharmacological inhibition of Akt efficiently abrogated both basal and TGF-β-stimulated Akt phosphorylation (Figure 2b).

To examine whether Akt is involved in TGF-β upregulation of collagen in dermal fibroblasts, the effects of Akt inhibitor on TGF-β-induced collagen protein levels were examined by western blot analyses. Treatment of cells with Akt inhibitor VIII or II did not prevent the induction of collagen by TGF-β (Figure 2c). The effect of inhibitor VIII on TGF-β-induced collagen steady-state mRNA levels was next examined. As shown before in Figure 1d, there was a marked downregulation in COL1A1 and COL1A2 basal mRNA levels, but the magnitude of TGF-β stimulation was similar in the presence or absence of the inhibitor (Figure 2d).

To determine whether Akt inhibition affects the Smad2/3 pathway, dermal fibroblasts were treated with increasing amounts of Akt inhibitor VIII in the presence or absence of TGF-β. Western blot analysis showed no effect of inhibitor on either basal or TGF-β stimulated levels of phospho-Smad3 and Smad2 (Figure 2e), consistent with the lack of effect of Akt on TGF-β-induced collagen synthesis. Taken together, these data suggest that in healthy dermal fibroblasts, Akt is not involved in the regulation of TGF-β-stimulated collagen synthesis.

Akt inhibition upregulates basal MMP1 levels and reverses TGF-β-induced inhibition of MMP1

Previous reports have shown that selected pharmacological inhibitors affected both collagen and MMP1 production (Fineschi *et al*., 2006; Poulalhon *et al*., 2006). Therefore, the effects of Akt inhibition on the expression of MMP1 were next studied. Dermal fibroblasts were treated with the Akt inhibitor VIII and MMP1 protein and mRNA levels were assessed by western blot and qRT–PCR. As shown in Figure 3a, Akt inhibition upregulated basal and TGF-β-induced MMP1 protein levels. The time course of the effects of TGF-β on MMP1 mRNA levels in the presence or absence of Akt inhibitor VIII is shown in Figure 3b. Consistent with previous findings (Edwards *et al*., 1987; Yuan and Varga, 2001; Yin *et al*., 2003), TGF-β treatment downregulated MMP1 levels as early as 3 hours, with significant decrease at 6 hours (50%). Akt blockade resulted in a time-dependent increase in MMP1 mRNA levels, with threefold upregulation at 12 hours and fourfold at 24 hours. Concomitant treatment with TGF-β and Akt inhibitor VIII reversed the inhibitory effect of TGF-β on MMP1, increasing the mRNA levels by 2.2-fold at 6 hours and by 3.8-fold at 24 hours. Taken together, these results suggest that Akt is a negative regulator of MMP1 gene expression in human dermal fibroblasts, and that it is required for the inhibitory effect of TGF-β.

Knockdown of Akt downregulates collagen and upregulates MMP1

Because of the known off target effects of inhibitors, we employed a second approach to block Akt. The three Akt isoforms (Akt_1, Akt_2, and Akt_3) are widely expressed and loss of one isoform can be partially compensated by the others, as demonstrated in animal models (Chen *et al*., 2001; Garofalo *et al*., 2003; Yang *et al*., 2004; Tschopp *et al*., 2005; Dummler *et al*., 2006). To block total Akt, specific small interfering RNAs (siRNAs) against Akt_1, Akt_2, and Akt_3 were concomitantly transfected into cells. There was similar inhibition efficiency for each isoform (80%), as assessed by qRT–PCR. In a separate experiment, each siRNA alone specifically downregulated its target gene, while having no effect on the other two isoforms (data not shown).

Next, the effects of Akt inhibition on COL1A1, COL1A2, and MMP1 mRNA levels were assessed by qRT–PCR. Following Akt knockdown, both chains of collagen were downregulated (50%) and MMP1 was upregulated (7 fold) (Figure 4). These results confirm that the effects of Akt inhibitors on collagen and MMP1 expression were specific.

Akt contributes to collagen upregulation in a TGF-βRI-based model of scleroderma

An increased ratio of TGF-βRI/TGF-βRII is a characteristic of SSc fibroblasts (Pannu *et al*., 2004). On the basis of this observation, we established an *in vitro* model of SSc by titration of an adenovirus expressing TGF-β type I receptor (AdTGF-βRI) in dermal fibroblasts (Pannu *et al*., 2006). Overexpression of TGF-βRI recapitulates the SSc phenotype, resulting in an increase in collagen type I and other profibrotic markers. We have demonstrated that in this model, collagen upregulation is independent of Smad3 activation (Pannu *et al*., 2007). To determine if Akt is activated in response to AdTGF-βRI overexpression, we analyzed its phosphorylation status by western blot at different time points after adenoviral transduction. Akt was activated in a time-dependent manner, starting at 6 hours

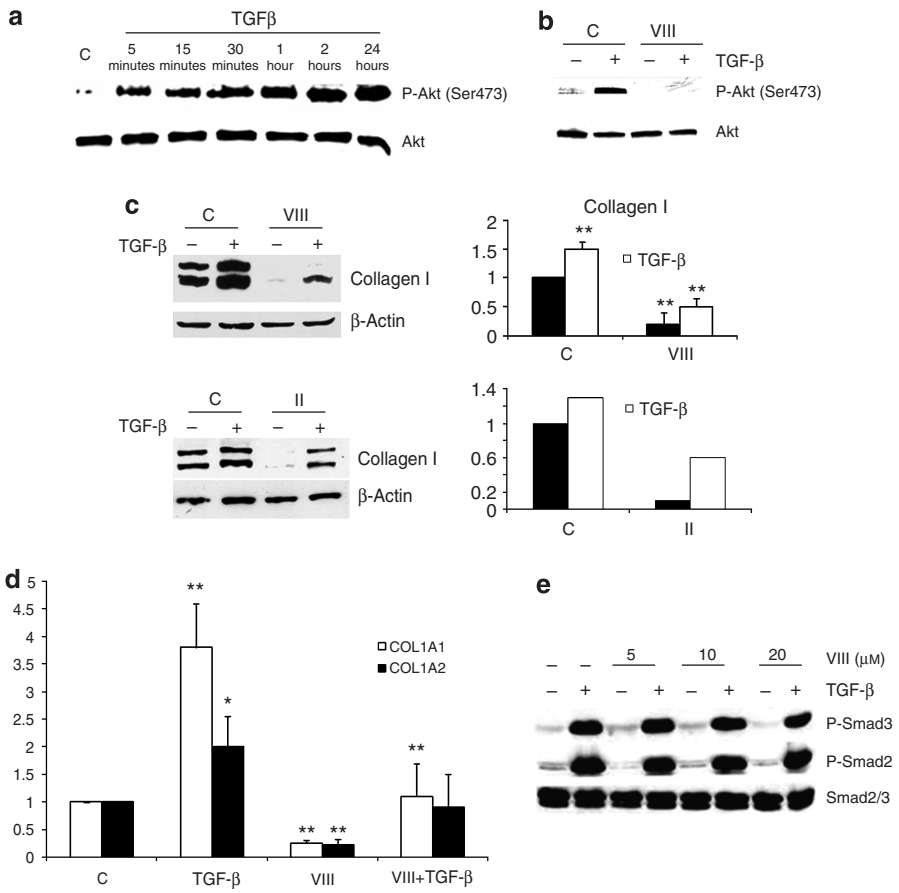

Figure 2. The effect of Akt inhibition on TGFβ-induced collagen gene expression. (**a**) Time-dependent phosphorylation of Akt in response to TGFβ. Serum-starved normal dermal fibroblasts were incubated in the presence of 2.5 ng ml^{-1} of TGFβ for the indicated time points. Levels of phospho-Akt (Ser473) were evaluated by western blot. The experiments were repeated two times, and representative data are shown. (**b**) Inhibition of basal and TGFβ-mediated Akt phosphorylation by Akt inhibitor VIII. Normal dermal fibroblasts were serum starved and treated with 20 μM of Akt inhibitor VIII or DMSO for 1 hour prior to stimulation with TGFβ (2.5 ng ml^{-1}) for 30 minutes. Phospho-Akt (Ser473) levels were evaluated by western blot. A longer exposure did not detect any bands in the presence of inhibitor. The experiments were repeated three times, and representative data are shown. (**c**) Decrease of basal and TGFβ-stimulated collagen protein levels with Akt inhibitors VIII and II. Normal dermal fibroblasts were treated with Akt inhibitor VIII (20 μM), II (5 μM) or DMSO for 1 hour prior to addition of 2.5 ng ml^{-1} TGFβ as indicated, and then at 48 hours cells were collected and analyzed by western blot for collagen. Representative data of three independent experiments are shown, with quantitative representation obtained by densitometric analysis. (**d**) The effect of Akt inhibitor VIII on TGFβ-stimulated COL1A1 and COL1A2 mRNA levels. Normal dermal fibroblasts were treated with 20 μM of Akt inhibitor VIII for 24 hours and then the mRNA levels were analyzed by qRT–PCR. mRNA values were normalized relative to control, DMSO-treated cells (arbitrarily set as 1) and means ± SEM of at least three independent experiments are shown. *$P<0.05$; **$P<0.01$ *versus* control, vehicle-treated cells. (**e**) Effects of Akt blockade on basal and TGFβ-stimulated phosphorylation of Smad2 and 3. Normal dermal fibroblasts were treated with different concentrations of the inhibitor VIII or DMSO for 1 hour and then incubated with 2.5 ng ml^{-1} TGFβ for additional 30 minutes. The levels of phospho-Smad2, phospho-Smad3, and total Smad2/3 were analyzed by western blot. The experiments were repeated two times and representative data are shown.

(and as early as 4.5 hours in a different experiment), and remained activated for at least 24 hours, with no change in the levels of total Akt (Figure 5a). As previously shown, the increase in TGF-βRI protein levels correlated with an increase in collagen production.

To determine if Akt is involved in collagen upregulation in this model, we treated cells with inhibitor II (1 μM) or VIII (10 μM). At this dose, treatment with Akt inhibitor II for 24 hours had little or no effect on the basal collagen levels, whereas completely preventing AdTGF-βRI-induced collagen synthesis. Similarly, treatment with inhibitor VIII resulted in a more efficient inhibition of collagen levels in cells over-expressing TGF-βRI than in control cells (Figure 5b). These data suggest that Akt is required for collagen upregulation in this model.

Akt is involved in increased collagen synthesis by SSc fibroblasts

The effects of Akt inhibition were compared in SSc fibroblasts *versus* normal controls. In a preliminary experiment, the minimal dose of inhibitor VIII required for significant inhibition of collagen in SSc was established as 10 μM for 24 hours. Under these experimental conditions, there was a more pronounced downregulation of collagen protein levels in SSc as compared with healthy fibroblasts. MMP1 protein levels were also more sensitive to the effects of Akt inhibition in SSc than in control fibroblasts (Figure 6a). Similar results

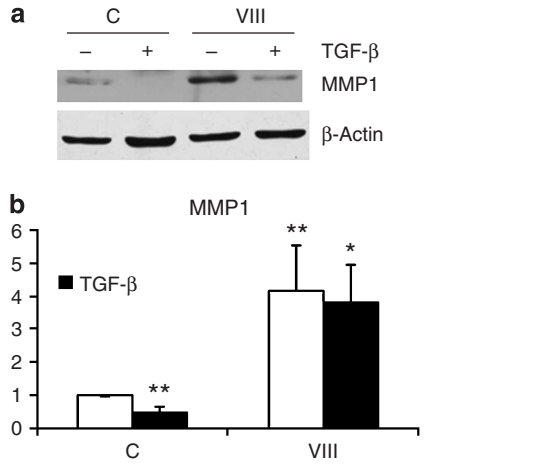

Figure 3. MMP1 levels are increased by Akt inhibition. (**a**) Normal dermal fibroblasts were treated with 20 μM Akt inhibitor VIII or DMSO, followed by addition of TGFβ (2.5 ng ml^{-1}) for 24 hours. MMP1 protein levels were analyzed by western blot. TGF-β-treated MMP1 protein levels are very low and only are detected after prolonged gel exposure. The experiments were repeated at least four times and representative data are shown. (**b**) Normal dermal fibroblasts were treated with Akt inhibitor VIII (20 μM) and TGF-β (2.5 ng ml^{-1}) for 24, and then MMP1 mRNA levels were analyzed by qRT–PCR. mRNA values were normalized relative to control, DMSO-treated cells (arbitrarily set as 1) and means ± SEM of at least three independent experiments are shown. *$P<0.05$; **$P<0.01$ *versus* control, vehicle-treated cells.

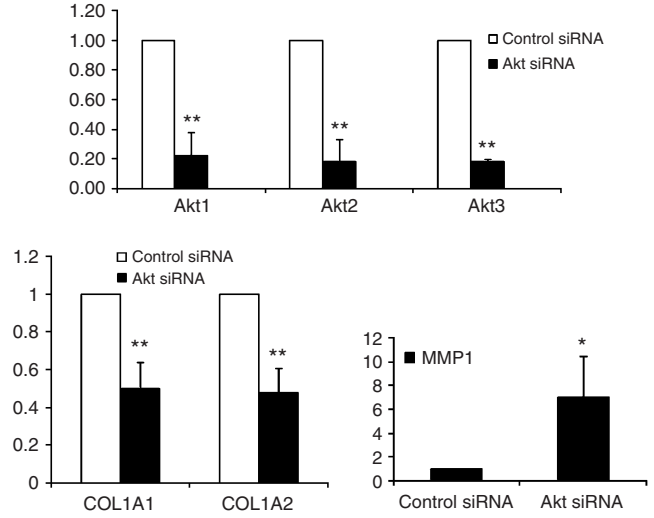

Figure 4. siRNA knockdown of Akt downregulates collagen and upregulates MMP1. Normal dermal fibroblasts were grown to 80% confluence and serum starved before transfection with 100 nM of Akt_1, Akt_2, and Akt_3, and the corresponding concentration of scrambled siRNA (Scrm). Twenty-four hours later, medium was changed to 10% fetal bovine serum and cells were harvested 48 hours after transfection. mRNA levels of Akt isoforms, collagen, and MMP1 were analyzed by qRT–PCR. Means ± SD of three independent experiments are shown, with values normalized to control (1). *$P<0.05$; **$P<0.01$ *versus* control, scrambled siRNA-treated cells.

were obtained at the mRNA levels by qRT–PCR (Figure 6c). Following Akt inhibition, SSc fibroblasts showed significant decrease in the mRNA levels of COL1A1 and COL1A2 (66 and 40% respectively). A less pronounced inhibition was

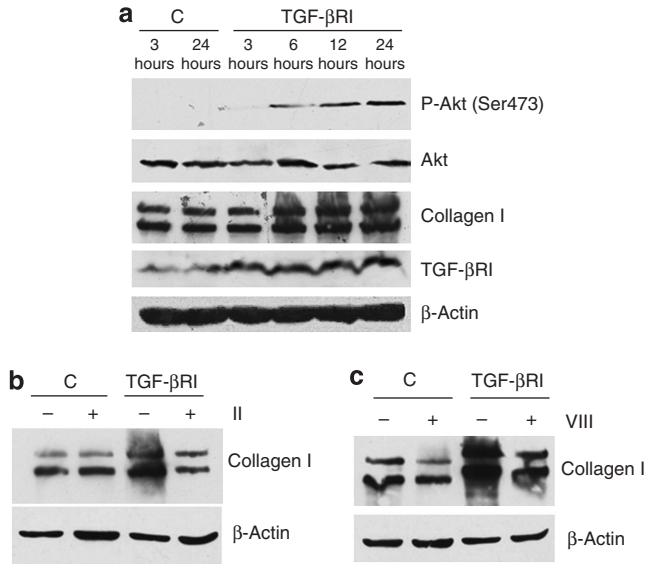

Figure 5. Contribution of Akt to collagen upregulation in the TGF-βRI-based model of scleroderma. (**a**) Effect of TGF-βRI overexpression on phospho-Akt and collagen levels. Normal dermal fibroblasts were transduced with AdTGF-βRI or AdGFP and harvested at the indicated time points. The levels of phospho-Akt (Ser473), Akt, TGFβRI, and collagen were examined by western blot. (**b**, **c**) Normal dermal fibroblasts were transduced with AdTGF-βRI in the presence or absence of Akt inhibitor II (**b**) or VIII (**c**) or DMSO, collected at 48 hours, and analyzed by western blot for collagen. β-Actin was used as control.

achieved in normal fibroblasts for COL1A1 and COL1A2 (25 and 10%, respectively). The increase in MMP1 levels with the inhibitor was 2.4-fold for SSc and twofold for healthy fibroblasts, but the differences were not statistically significant.

Published data suggest that Akt activation is involved in the increased myofibroblast transdifferentiation seen in SSc (Laplante *et al.*, 2005). There was a significant decrease (45%) in α-smooth-muscle actin (α-SMA) mRNA levels in response to Akt inhibitor in SSc fibroblasts (Figure 6c), further confirming this hypothesis. Although levels of α-SMA in healthy cells were also decreased (by 40%), the results were not statistically significant (Figure 6c). Taken together, these results suggest that with respect to profibrotic markers, cultured SSc fibroblasts show increased sensitivity to Akt inhibition.

DISCUSSION

This study examined the role of Akt in matrix regulation in human dermal fibroblasts and its contribution to the SSc phenotype. We show that Akt regulates collagen and MMP1 production in an opposite manner. Using several approaches, including Akt inhibitors, Akt siRNA, and constitutively active and dominant-negative Akt constructs, we demonstrated that Akt upregulates collagen type I gene expression at the protein, mRNA, and promoter level in human dermal fibroblasts. By similar means we showed that MMP1 mRNA and protein levels are negatively regulated by Akt. In addition, we provide evidence that Akt signaling contributes

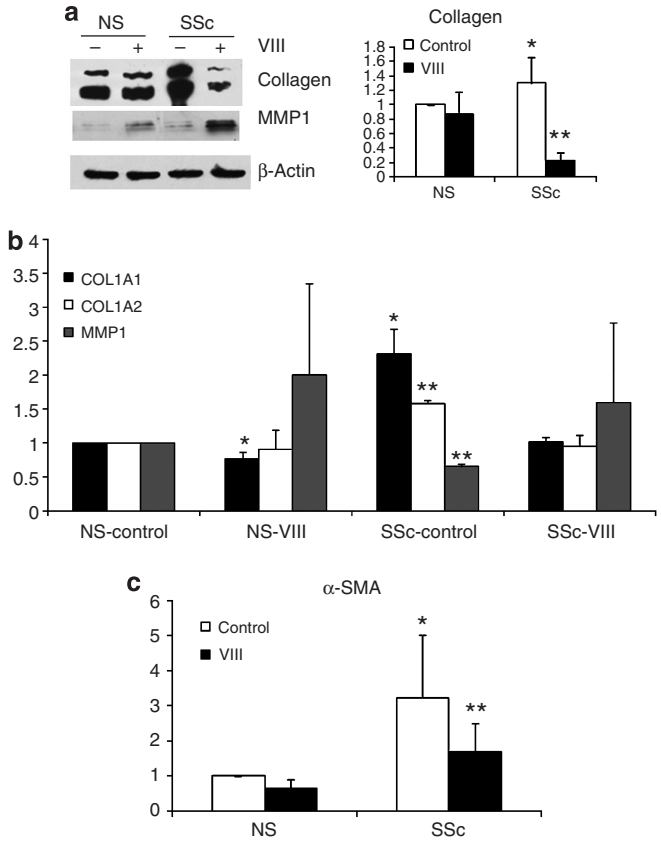

Figure 6. Akt contributes to collagen upregulation in SSc fibroblasts. SSc and matched control fibroblasts were incubated in the presence or absence of half of the optimal dose established for normal fibroblasts of Akt inhibitor VIII (10 μM) for 24 hours and then the protein (**a**) and mRNA (**b**) levels of collagen and MMP1 and the mRNA levels of α-SMA (**c**) were analyzed by western blot and qRT–PCR, respectively. Representative data are shown in (**a**) and means ± SD of three independent experiments are shown, with values normalized to NS control (1) in (**b**) and (**c**). *$P<0.05$; **$P<0.01$ *versus* NS control, vehicle (DMSO)-treated cells.

to the fibrotic SSc phenotype and that SSc fibroblasts show enhanced sensitivity to Akt blockade.

Akt is a central element in the PI3K/Akt network, and there is increasing evidence that this pathway plays a role in fibrosis. The importance of PI3K in collagen gene regulation was previously demonstrated in human dermal fibroblasts (Asano *et al.*, 2004), lung fibroblasts (Ricupero *et al.*, 2001), hepatic stellate cells (Reif *et al.*, 2003), and mesangial cells (Runyan *et al.*, 2004). In addition, multiple downstream targets of PI3K have previously been linked to collagen regulation, including Akt, mammalian target of rapamycin (Shegogue and Trojanowska, 2004; Poulalhon *et al.*, 2006), PKC-δ ((Jimenez *et al.*, 2001), extracellular signal-regulated kinase (Pannu *et al.*, 2007), and pp70-ribosomal S6 kinase (Gabele *et al.*, 2005). In mesangial cells, Akt was required for induction of collagen by mechanical stretch (Krepinsky *et al.*, 2005) and by high glucose levels (Wu *et al.*, 2007). Akt is constitutively phosphorylated in cells derived from fibrotic areas in a mouse model of bleomycin-induced pulmonary fibrosis (Vittal *et al.*, 2005). In a recent study, analysis of skin from *Akt1−/−* mice revealed an impaired matrix organization with reduced amount of collagen and decreased density of collagen fibrils, suggesting the contribution of this pathway to collagen synthesis *in vivo* (Chen *et al.*, 2005). Interestingly, in a recent report, PI3K activation in response to TGF-β resulted in independent activation of p21 (CDKN1A)-activated kinase 2 and Akt in a fibroblast-specific manner (Wilkes *et al.*, 2005). p21 (CDKN1A)-activated kinase 2 activation in these cells was also shown to mediate the activation of abelson tyrosine kinase (c-Abl) a target of the antifibrotic drug imatinib (Wilkes and Leof, 2006). Although the exact roles of Akt and c-Abl in fibrosis are not yet known, the proposed model could explain distinct pathways through which PI3K regulates collagen synthesis in fibroblasts.

TGF-β is a central mediator of collagen deposition and a major player in SSc fibrosis. There is evidence that PI3K/Akt can be activated downstream of TGF-β and a direct interaction between the TGF-β receptor complex and PI3K was also demonstrated (Wilkes *et al.*, 2005; Yi *et al.*, 2005). Furthermore, a recent study has shown that in fibroblasts, PI3K activation in response to TGF-β does not affect Smad2/3 phosphorylation, nuclear translocation and transcriptional activity (Wilkes *et al.*, 2005). In agreement with this, our data show that in human dermal fibroblasts, Akt inhibition does not affect Smad2/3 activation. Although at this point we cannot exclude a PI3K/Akt-mediated regulation of Smad phosphorylation in the linker region (Asano *et al.*, 2004; Runyan *et al.*, 2004), this report confirms that TGF-βRI-induced Smad phosphorylation is not dependent on Akt activation. Although several previous studies reported that PI3K is involved in TGF-β regulation of collagen gene expression, in our study Akt blockade did not prevent TGF-β induction of collagen, suggesting that the effects of TGF-β on collagen are mediated through activation of distinct PI3K effectors. Thus, while inhibiting the components of the PI3K network ultimately leads to decreased collagen deposition by fibroblasts, distinct cellular mechanisms may be involved in mediating this effect.

MMP1 is an interstitial collagenase secreted by fibroblasts and other cells, that catalyses collagen degradation. Reduced MMP1 expression is a characteristic of SSc fibroblasts and may contribute to excessive collagen deposition seen in patients with SSc (Takeda *et al.*, 1994). Our results demonstrate that Akt inhibition leads to increased MMP1 mRNA and protein. In addition, blockade of Akt in the presence of TGF-β resulted in a very potent upregulation of MMP1 protein and mRNA levels, reversing the inhibitory effect of TGF-β on MMP1 gene expression. Thus, although Akt is not involved in the TGF-β induced upregulation of collagen, it is required for the TGF-β-mediated suppression of MMP1. In agreement with our findings, several reports have shown that an upstream Akt activator, insulin growth factor 1 (IGF-1), is a negative regulator of MMP1 expression (Canalis *et al.*, 1995; Hui *et al.*, 2001; Im *et al.*, 2003). Furthermore, inhibition of another Akt activator, vascular endothelial growth factor (VEGF), resulted in downregulation of MMP1 (Kamochi *et al.*, 2002). Consistent with our study showing dual effects of Akt on collagen and MMP1 production,

inhibition of mammalian target of rapamycin (mTOR) by rapamycin also induced collagen gene expression while having an opposite effect on MMP1 (Poulalhon *et al.*, 2006). Thus, mammalian target of rapamycin, as a downstream Akt effector, could contribute to the effects of Akt on collagen and MMP1. Similar dual effects on MMP1 and collagen expression were recently described using proteasome blockade (Fineschi *et al.*, 2006). Several studies have linked proteasome blockade with Akt inhibition via upregulation of protein phosphatase 2A (PP2A) (Wei and Xia, 2006) or phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Fujita *et al.*, 2006), suggesting that the specific effects of these inhibitors on collagen and MMP1 could be at least in part mediated via downregulation of the Akt pathway.

Systemic sclerosis biopsies are characterized by the presence of constitutively phosphorylated Akt, but the mechanism leading to its activation is currently unknown. One of the main pathways that are upregulated in SSc *in vivo* is the TGF-β pathway. Emerging evidence suggests that in addition to canonical Smad activation, TGF-β induces parallel signaling pathways that may modulate or be independent of Smad signaling (Bakin *et al.*, 2000). The PI3K/Akt pathway is among the non-Smad signaling mediators that have been shown to be activated by TGF-β (Dumont *et al.*, 2003; Jun *et al.*, 2005). Furthermore, a previous report indicated that constitutively active TGF-βRI overexpression potently activated PI3K in epithelial cells (Yi *et al.*, 2005). Increased TGF-βRI expression levels are a characteristic feature of SSc fibroblasts and were shown to contribute to the elevated matrix gene expression in these cells (Pannu *et al.*, 2004). We have previously established an *in vivo* model on the basis of adenovirus-mediated TGF-βRI overexpression, and showed that this model recapitulates the SSc phenotype with respect to elevated matrix production (Pannu *et al.*, 2006). In addition, we found that collagen upregulation in response to aberrant TGF-βRI signaling is independent of Smad2/3 and dependent on Smad1 and extracellular signal-regulated kinase (Pannu *et al.*, 2007). Furthermore, we now show that in this model Akt is activated at early time points, suggesting that aberrant TGF-βRI signaling in SSc could contribute to increased Akt phosphorylation seen in this disease. We also provide evidence that Akt is required for AdTGF-βRI-dependent upregulation of collagen. Further studies are necessary to elucidate the mechanism of collagen regulation by Akt in this model.

To test the potential clinical importance of Akt activation in SSc, we evaluated the sensitivity of cultured SSc fibroblasts to Akt blockade. Collagen downregulation in SSc fibroblasts was achieved after exposure for a shorter time at lower doses of Akt inhibitor than the ones used to obtain similar responses in normal fibroblasts. Furthermore, these conditions failed to inhibit collagen in the normal, matched control fibroblasts. Increased levels of α-SMA were reported in SSc *in vitro* and *in vivo* (Kirk *et al.*, 1995; Jelaska and Korn, 2000). Coexpression of α-SMA and phosphorylated Akt was reported in SSc *in vivo* (Jun *et al.*, 2005) and PI3K/Akt activation was previously linked to myofibroblasts differentiation (Laplante *et al.*, 2005). In agreement with this, our results show that Akt positively regulates the expression of α-SMA mRNA in SSc fibroblasts. The increased sensitivity seen with SSc fibroblasts identifies Akt as a potential target for the antifibrotic therapy in SSc.

MATERIALS AND METHODS

Reagents

The pharmacologic inhibitors Akt inhibitor VIII and II, protease inhibitor cocktail set III, and phosphatase inhibitor cocktail set II were purchased from Calbiochem (San Diego, CA). Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). Tissue culture reagents, DMEM and 100 × antibiotic antimycotic solution (penicillin streptomycin and amphotericin B) were obtained from Gibco BRL (Grand Island, NY) and fetal bovine serum was purchased from HyClone (Logan, UT). Enhanced chemiluminescence reagent and bicinchoninic acid protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). TRI Reagent was purchased from the Molecular Research Center Inc. (Cincinnati, OH).

Antibodies used were as follows: goat anti-type I collagen (Southern Biotechnology, Birmingham, AL); monoclonal β-actin, clone AC-150 (Sigma, St Louis, MO), MMP1 (Chemicon, Temecula, CA); Akt, phospho-Akt (Ser473), Smad2, Smad3, and Smad2/3 (Cell Signaling); and TGF-βRI (Santa Cruz Biotechnology, Santa Clara, CA). Primers were obtained from Operon (Huntsville, AL).

Cell culture

Human fibroblasts were obtained from skin biopsies from the dorsal forearm of healthy donors or patients who had diffuse cutaneous SSc for less than a year, upon informed consent and in compliance with the Institutional Review Board for Human Studies. The study was conducted according to the Declaration of Helsinki Principles. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for SSc (American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980) and had not undergone any treatment for SSc at the time of biopsy. Biopsy specimens from healthy donors matched with each SSc patient for age, sex, and race were processed in parallel.

Dermal fibroblasts were cultured from the biopsy specimens as described previously (Pannu *et al.*, 2007).

Adenoviral constructs

Replication-incompetent adenoviral vectors expressing rat full-length ALK5/TGF-βRI (AdTGF-βRI) and control green fluorescent protein (AdGo) were generated as described earlier (Pannu *et al.*, 2004). The dose used to transducer dermal fibroblasts was 50 multiplicities of infection of the adenovirus.

Plasmid constructs, transient transfection, and CAT assay

The −772*COL1A2/CAT* construct contains the human *COL1A2* fragment from +58 to −772 bp fused to the CAT reporter gene (Ihn *et al.*, 1996). Expression vectors containing constitutively active and dominant-negative *Akt* expressed from the *CMV* promoter have been previously described (Bellacosa *et al.*, 1998). All transfections were performed using FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The pSV-β-galactosidase control vector (Promega, Madison, WI) was co-transfected with the collagen promoter and

used to normalize for transfection efficiency. Cells were stimulated 24 hours after transfection with TGF-β and incubation was continued for another 24 hours. Then cells were scraped and lysed in lysis buffer (Promega). CAT activities were measured as described previously (Ihn *et al.*, 1996).

siRNA experiments

SMARTpool siRNA directed against human Akt_1, Akt_2, and Akt_3 were purchased from Dharmacon RNA Technologies, CO. Negative-control siRNA was purchased from Qiagen (Chatsworth, CA) and RNAiFect™ (Qiagen) was used for transfection of dermal fibroblasts according to the manufacturer's recommendations.

Immunoblotting

Dermal fibroblasts were grown to confluence, serum-starved for 24 hours, and then subjected to different treatments. After the appropriate time period, the medium was removed and cells were processed as described previously (Pannu *et al.*, 2004).

Total cellular RNA extraction, cDNA preparation, and quantitative real-time RT–PCR analysis

Total RNA was extracted using TRI Reagent. Quality was assessed by agarose-gel method and (~)1 μg of RNA was used to prepare cDNA using Transcriptor First-Strand synthesis kit (Roche Applied Sciences, Indianapolis, IN). Real-time RT–PCR was performed using IQ SYBR Green mixture (Bio-Rad, Hercules, CA) on an iCycler PCR machine (Bio-Rad) using 1 μl of cDNA in triplicate with β2-microglobulin as internal control. Side-strand-specific primers for COL1A1, COL1A2, β2-microglobulin, MMP1, Akt_1, Akt_2, Akt_3, and α-SMA were as follows: COL1A1 forward (CCAGAAGAACTGGTACATCAGCA), COL1A1 reverse (CGCCATACTCGAACTGGGAAT); COL1A2 forward (GATGTTGAACTTGTTGCTGAGG), COL1A2 reverse (TCTTTC CCCATTCATTTGTCTT); β2-microglobulin forward (GCCGTGTGAA CCATGTGACTTT), β2-microglobulin reverse (CCAAATGCGGCATC TTCAAA); MMP1 forward (TCTGGGGTGTGGTGTCTA), MMP1 reverse (GCCTCCCATCATTCTCAGGTT); Akt_1 forward (CGTGACC ATGAACGAGTTTG), Akt_1 reverse (GCCACGATGACTTCCTTCTT); Akt_2 forward (ACGTGGATTCTCCAGACGA), Akt_2 reverse (GCTGCT TGAGGCTGTTGG); Akt_3 forward (TGGATTTACCTTATCCCCTCAA), Akt_3 reverse (TGGCTTTGGTCGTTCTGTTT); and α-SMA forward (GCACTGCCTTGGTGTGTG), α-SMA reverse (TCCCATTCCCAC CATCAC).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was funded through National Institute of Health grant AR-44883 to Maria Trojanowska and through an Arthritis Foundation postdoctoral fellowship to Andreea M. Bujor.

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