Published in final edited form as:

Exp Dermatol. 2010 April ; 19(4): . doi:10.1111/j.1600-0625.2010.01065.x.

Akt inhibition up-regulates MMP1 through a CCN2-dependent pathway in human dermal fibroblasts

Andreea M. Bujor^{*}, Sashidar Nakerakanti^{*}, Erin Morris, Faye N. Hant, and Maria Trojanowska^{*}

Division of Rheumatology & Immunology, Medical University of South Carolina, Charleston, SC, USA

Abstract

Akt is a key signalling molecule that was found to be down-regulated in chronic wounds. Akt blockade has dual antifibrotic effects in human dermal fibroblasts, by up-regulating matrix metalloproteinase 1 (MMP1) and down-regulating collagen gene expression (J Invest Dermatol 2008; 128: 1906). The aim of this study was to gain additional insights into the mechanism of MMP1 up-regulation following Akt blockade. As previous studies showed that CCN2 can be a positive regulator of MMP1, we examined the effects of Akt inhibition on CCN2 expression. Akt blockade using a specific pharmacological inhibitor and Akt siRNA resulted in a significant up-regulation of CCN2, which correlated with the increase in MMP1. The MMP1 up-regulation following Akt blockade was partially suppressed by CCN2 siRNA, suggesting that CCN2 is contributing to this effect. Additional experiments showed that CCN2 induces phosphorylation of ERK1/2, Ets1 and c-Jun. Consistent with the stimulatory role of ERK1/2/Ets1 in the expression of MMP1, the ERK1/2 inhibitor UO126 prevented the phosphorylation of ERK1/2 and Ets1 and completely abrogated the induction of MMP1 after CCN2 overexpression, while having no effect on c-Jun activation. Taken together these results establish CCN2 as a key regulator of MMP1 induction via activation of the ERK1/2/Ets1 pathway. Down-regulation of Akt signalling leads to inappropriate activation of the CCN2/MMP1 pathway that may contribute to the pathogenesis of chronic wounds. Coordinate expression of CCN2, Akt and MMP1 could be important for normal wound healing to occur. Thus, targeting these specific proteins may represent a promising approach to the therapy of dysregulated wound healing.

Keywords

Akt; extracellular matrix; CCN2; MMP1

Introduction

The process of wound healing consists of a series of coordinated events aimed at restoring tissue integrity. It is characterized by three partially overlapping phases: the inflammatory, proliferative and remodelling phase. In the first phase, inflammatory cells invade the wound, begin the phagocytosis of cell debris and release various growth factors and cytokines, which initiate the next phase of wound repair. Starting around day 5, the proliferative phase begins with the migration and proliferation of keratinocytes, fibroblasts and endothelial cells

© 2010 John Wiley & Sons

Correspondence: Andreea M. Bujor, MD, Arthritis Center - Rheumatology, Boston University Medical Campus, 72 East Concord Street, Evans 501, Boston, MA 02118, USA, Tel.: + 617 638 7503, Fax: + 617 638 5226, andreea@bu.edu.

**Current address:* Arthritis Center, Boston University Medical Campus, Boston, MA, USA

in the wounded area. Fibroblasts become activated and contribute to the repair of dermis around the wound by laying down abundant extracellular matrix (ECM) and by transforming into myofibroblasts that are responsible for wound contraction. The formation of provisional matrix is accompanied by massive angiogenesis and nerve sprouting and the resulting new tissue is called granulation tissue. The final phase of wound healing begins after 2–3 weeks and can last for years, depending on the severity of the injury. Transition from granulation to mature scar tissue occurs, characterized by continued synthesis and remodelling of ECM proteins through the action of various matrix metalloproteinases and serine proteases (1).

Akt (acutely transforming retrovirus AKT8 in rodent T-cell lymphoma), also referred to as protein kinase B, is a serine threonine kinase with critical roles in many biological processes including cell survival, proliferation, migration, angiogenesis and metabolism. It is activated downstream of phosphatidylinositol 3-kinase (PI3K) (2) by a plethora of growth factors and cytokines, including transforming growth factor (TGF). Three different isoforms have been described: Akt₁, Akt₂ and Akt₃, that are very similar in their structure and may regulate distinct biological processes (3). A growing body of evidence points to the central role of Akt in skin biology in general and in wound healing in particular. Upregulated levels of Akt were reported in exaggerated wound-healing responses (4), while decreased Akt signalling was linked to chronic, non-healing wounds (5). Studies in our laboratories showed that Akt blockade has dual antifibrotic effects, increasing the synthesis of matrix metalloproteinase 1 (MMP1) and reducing the production of type I collagen (6). However, the mechanism of MMP1 upregulation in response to Akt inhibition and its functional significance are presently unknown.

Connective tissue growth factor (CCN2) is a secreted protein, a member of the CNN family of matrix-associated proteins encoded by immediate early genes that plays important roles in embryonic development, differentiation, angiogenesis, chondrogenesis and wound healing. CCN2 is an important mediator of TGF in the induction of type I collagen and is overexpressed in various types of fibrotic diseases, thus being considered a major player in fibrosis (7). However, according to recent studies, the role of CCN2 in the regulation of ECM could be more complex. Several reports have implicated CCN2 as a positive regulator of different matrix metalloproteinases, including MMP1 (8–10), but the mechanism of CCN2-induced MMP1 up-regulation has not yet been adequately addressed.

MMP1 is an interstitial collagenase, a member of the family of matrix metalloproteinases that has the ability to degrade fibrillar collagen molecules, including type I and type III collagens. It is involved in numerous biological and pathological processes and it was shown to play important roles in wound healing (11). MMP1 expression is regulated at the transcriptional level by a variety of cytokines and growth factors, such as tumor necrosis factor alpha (TNF) and interleukin-1 (IL-1) (12), through activation of the activator protein-1 (AP-1) and Ets (E-twenty-six) family of transcription factors, which cooperate in the induction of MMP1 gene expression. Members of the mitogen-activated protein kinase (MAPK) pathways are important mediators of these processes (13–15). Tight control of MMP1 activity by tissue inhibitors of matrix metalloproteinases is important to prevent inappropriate, excessive matrix degradation. Elevated MMPs have been implicated in the pathogenesis of chronic wounds of diabetic and venous insufficiency ulcers (16–19) and several independent investigators have shown decrease of MMP1 in fibroblasts from hypertrophic scars (20,21).

In this study, we examined the mechanism of CCN2-induced MMP1 up-regulation and its relationship with Akt blockade in human dermal fibroblasts. Our results show that CCN2 is required for the MMP1 induction following Akt inhibition. We also demonstrate that CCN2 induces MMP1 expression via an extracellular signal-regulated kinase 1/2 (Erk1/2)/Ets1-

dependent pathway. Given the important roles of Akt, CCN2 and MMP1 in wound healing, a better understanding of the relationship between these molecules may enable us to develop new therapeutic strategies for the treatment of aberrant wound healing.

Methods

Materials

Recombinant human CCN2 was obtained from ProSpecTany TechnoGene (Rehovot, Israel) and EMP Genetech (Ingolstadt, Germany) and the pharmacological inhibitors Akt inhibitor VIII, protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II were purchased from Calbiochem (San Diego, CA, USA). TGF- β 1 was from R&D Systems (Minneapolis, MN, USA). Tissue culture reagents, Dulbecco's modified eagle medium (DMEM) and the antibiotic antimycotic solution (penicillin, streptomycin and amphotericin B) were obtained from Gibco BRL (Grand Island, NY, USA) and foetal bovine serum was purchased from HyClone (Logan, UT, USA). Dimethyl sulfoxide (Me₂SO) was obtained from Sigma (St. Louis, MO, USA). For RNA isolation, TRI Reagent was purchased from the Molecular Research Center Inc. (Cincinnati, OH, USA). c-DNA was synthesized using Transcriptor First Strand synthesis kit (Roche, Indianapolis, IN, USA) and quantitative real time RT-PCR (qRT-PCR) assays were performed using MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad iCycler, Hercules, CA, USA) as previously described (6). Primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL, USA). The following antibodies were used: monoclonal α -actin antibody, clone AC-150 (Sigma); CCN2 antibody, clone L-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); MMP1 antibody – MAB3307 from Chemicon International (Temecula, CA, USA); antitype I collagen (Southern Biotechnology, Birmingham, AL, USA); ERK1/2, phospho-ERK1/2 (Thr202/Tyr 204) and Akt were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). enhanced chemiluminescent substrate (ECL) reagent and anti-rabbit horseradish peroxidase-linked whole antibody (from donkey) were obtained from Amersham Biosciences (Piscataway, NJ, USA). Bicinchoninic acid protein assay reagent was from Pierce.

Cell culture

Human dermal fibroblasts were established from biopsy specimens obtained from the dorsal forearm of healthy volunteers after informed consent. Tissue was dissociated enzymatically by 0.25% collagenase type I (Sigma) and 0.05% DNase (Sigma) in Dulbecco's modified Eagle's medium with 20% foetal bovine serum overnight at 37°C. Primary fibroblast cultures were established in 25-cm² culture flasks in DMEM supplemented with 20% fetal calf serum (FCS) and 2 mM glutamine. Monolayer cultures were maintained at 37°C in 10% CO₂ until confluence. Cells were then passaged and used for experiments.

Western blot analysis

Human dermal fibroblast cultures were serum-starved overnight and then treated as described. Cells were harvested and lysed in a buffer containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), 1% Nonidet P-40, 15 mM NaF, 1 mM β -mercaptoethanol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin. Protein concentration was quantified using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). To ensure equal loading for conditioned media, cells were trypsinized and counted. Corresponding amounts of conditioned media or fifty micrograms of cell lysates were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with the indicated primary antibodies overnight in 2% milk at a dilution of 1:1000, then washed and incubated for 1 h with the corresponding secondary antibody.

Bands were detected using ECL (Pierce). Position of the reference molecular weight markers is shown in figures at the left of each blot.

RNA isolation, cDNA preparation, and qRT-PCR analysis

Total RNA was extracted using TRI Reagent as previously described (6). Total RNA (1 µg) was converted to cDNA using random hexamer primers and with the aid of Transcriptor First Strand synthesis kit (Roche). qRT-PCR was performed using IQ SYBR Green mixture (Bio-Rad) on an iCycler PCR machine (Bio-Rad) using 1 µl of cDNA in triplicate with 2-microglobulin as internal control. The following primers were used: 2-microglobulin forward (GCC GTG TGA ACC ATG TGA CTT T), 2-microglobulin reverse (CCA AAT GCG GCA TCT TCA AA); MMP1 forward (TCT GGG GTG TGG TGT CTA), MMP1 reverse (GCC TCC CAT CAT TCT CAG GTT); CCN2 forward (TTG CGA AGC TGA CCT GGA AGA GAA) and CCN2 reverse (AGC TCG GTA TGT CTT CAT GCT GGT).

Inhibition of protein expression by small interfering RNA (siRNA)

For the inhibition of Akt expression using specific siRNA reagents, dermal fibroblasts were grown to 80–90% confluence, serum starved for 4 h and transiently transfected with 20 µM of Akt₁, Akt₂, and Akt₃, or the corresponding concentration of scrambled non-silencing siRNA (Scrm). Twenty-four hours later, medium was changed to 10% fetal bovine serum and cells were harvested 72 h after transfection.

Adenoviral constructs

Replication-incompetent adenoviral vectors expressing human full-length CCN2 (AdCCN2) and control green fluorescent protein (AdGo) were generated as described earlier (22). The dose used to transduce dermal fibroblasts was 50 multiplicities of infection of the adenovirus.

Results

CCN2 up-regulation stimulates MMP1 production by human dermal fibroblasts

In contrast to its known profibrotic role downstream of TGF β , several reports have suggested that CCN2 could also play a role in matrix remodeling (8–10). To investigate the role of CCN2 in MMP1 gene regulation dermal fibroblasts were transduced with increasing doses of the previously described full-length CCN2 over-expressing adenovirus (22) for 72 h and then the levels of CCN2, MMP1 and collagen type I were analysed by Western blot. As seen in Fig. 1a, the increased levels of secreted MMP1 paralleled those of CCN2. Similarly, treatment of cells with recombinant human CCN2 up-regulated MMP1 levels in a dose-dependent manner (Fig. 1b). Interestingly, very high levels of CCN2 resulted in a significant down-regulation of collagen type I protein levels, both after adenoviral-mediated over-expression and the use of recombinant protein.

Akt inhibition up-regulates basal and TGF β -induced CCN2 levels

Our recent studies have shown that Akt is a negative regulator of MMP1 gene expression in human dermal fibroblasts, while having a positive role in collagen synthesis (6). We next examined the effects of Akt inhibition on CCN2 expression. Dermal fibroblasts were treated with increasing amounts of Akt inhibitor VIII, and the protein levels of CCN2 were evaluated 48 h later by Western blot. As seen in Fig. 2a, there was a potent induction of CCN2 with the smallest dose of inhibitor tested, suggesting that Akt is a negative regulator of CCN2 expression in human dermal fibroblasts.

The increase in CCN2 protein levels after Akt inhibition may occur via transcriptional or posttranscriptional mechanisms. The expression of CCN2 following Akt blockade was further analysed by qRT-PCR. Normal dermal fibroblasts were exposed to 20 μM of Akt inhibitor VIII, and the mRNA levels of CCN2 were measured after different time points. The increase in CCN2 was time dependent, with a significant up-regulation starting at 1 h and a peak at 6 h, followed by a reduction in the mRNA levels after 12 h of treatment (Fig. 2b). Therefore, our results suggest that transcriptional control plays a relatively minor role in the prolonged up-regulation of CCN2 protein levels after Akt inhibition. Further studies will be needed to define the exact mechanism of Akt-mediated CCN2 regulation.

To rule out the non-specific effects that pharmacological inhibitors may exhibit, we depleted endogenous levels of Akt using a combination of Akt₁, Akt₂ and Akt₃ siRNA. As seen in Fig. 2c, siRNA-mediated down-regulation of Akt resulted in a significant induction of CCN2 protein levels. The control siRNA had no significant effect on the expression of CCN2 (data not shown).

We next investigated the relationship between MMP1 and CCN2 up-regulation following Akt inhibition. As shown in Fig. 2d, blockade of Akt signalling using increasing doses of Akt inhibitor VIII resulted in a dose-dependent up-regulation of CCN2 protein levels that was paralleled by an increase in MMP1 expression, suggesting a link between these two events.

TGF β is a potent inducer of CCN2 gene expression and it has been long postulated that CCN2 is required for the TGF β -induced collagen synthesis. In contrast to its positive role in the regulation of CCN2, TGF β is known to down-regulate MMP1 gene expression. We next sought to examine the effects of Akt blockade on CCN2 and MMP1 expression in the presence of TGF β . Prior to growth factor treatment, confluent dermal fibroblasts were treated with 20 μM of Akt inhibitor VIII and then 72 h later cell pellets were collected and CCN2 protein levels were analysed by Western blot. Confirming the data presented in Fig. 2a, Akt inhibition resulted in a significant up-regulation of CCN2 protein, to levels similar to those obtained after TGF β treatment. In addition, induction of CCN2 by Akt inhibition was accompanied by a potent up-regulation of MMP1. Interestingly, Akt blockade cooperated with TGF β in the induction of CCN2 and completely reversed the inhibitory effect of TGF β on MMP1 gene expression (Fig. 2e).

We concluded from these experiments that Akt negatively regulates CCN2 synthesis both at the basal level and in response to TGF β .

CCN2 mediates MMP1 up-regulation in response to Akt blockade

As previous studies have implicated CCN2 in MMP1 regulation (8–10), we next investigated whether CCN2 overexpression contributes to the MMP1 induction in response to Akt blockade. Consistent with our previous results in Fig. 1a and with published data (8–10), adenovirus-mediated CCN2 over-expression resulted in an up-regulation of MMP1, similar to Akt inhibition. However, higher levels of CCN2 were required to obtain similar effects on MMP1 expression at the basal levels than the levels reached after Akt inhibition (Fig. 3a), suggesting that Akt activation status could modulate the levels of MMP1 via additional mechanisms. To confirm the role of CCN2 in the induction of MMP1 in response to Akt blockade, we down-regulated the levels of CCN2 using the siRNA method. As seen in Fig. 3b, efficient suppression of CCN2 protein was achieved using adenovirus-mediated CCN2 specific siRNA, both at the basal level and in TGF β and Akt inhibitor VIII-treated cells. Akt inhibition resulted in a potent up-regulation of MMP1 protein levels, that was partially blocked (approximately 50%) by siRNA to CCN2, and the results were statistically

significant. Together, these results suggest that up-regulation of MMP1 following Akt blockade requires CCN2 induction.

Inhibition of Akt signalling induces basal and enhances TGF β -mediated Erk1/2 phosphorylation

Published data suggest that ERK1/2 can be activated in response to inhibited Akt signalling (23–26), therefore we next sought to determine whether Akt blockade contributes to ERK1/2 phosphorylation in human dermal fibroblasts. To test this, we used an adenoviral vector overexpressing a dominant negative form of Akt₁ and transduced the cells in the presence and absence of TGF β for different time points. As shown in Fig. 4a, there was a potent stimulation of Erk starting at 15 min after TGF β , followed by a second peak at 6 h. Adenovirus-mediated overexpression of a dominant negative form of Akt₁ resulted in significant ERK1/2 phosphorylation and had a synergistic effect with TGF β in the induction of ERK1/2 activation. This suggests that in human dermal fibroblasts, Akt blockade induces sustained ERK1/2 phosphorylation.

CCN2-induced MMP1 gene expression is mediated via Erk1/2 and Ets1 in human dermal fibroblasts

Activation of ERK1/2 downstream of CCN2 was shown in human chondrocytes (27) and published evidence links ERK1/2 with the regulation of MMP1 promoter via AP1 and Ets sites (15,28). To gain insight into the mechanism of CCN2-induced MMP1 up-regulation, we next examined whether CCN2 affects the activation status of several major signalling molecules implicated in MMP1 gene expression. Recombinant human CCN2 induced Ets1 phosphorylation starting with the smallest dose tested (10 ng/ml, data not shown). A time course analysis of extracts from human dermal fibroblasts treated with recombinant human CCN2 revealed an increase in the levels of phosphorylated Ets1 starting at 15 min that was maintained after 6 h (Fig. 4b). Consistent with data obtained in chondrocytes, CCN2 treatment was accompanied by ERK1/2 phosphorylation with a peak at 15 min that was maintained up to 6 h, similar to Ets1 activation.

To investigate whether CCN2-induced up-regulation of MMP1 is mediated via activation of ERK1/2/Ets1, we pretreated cells with UO126, a specific inhibitor of MEK (MAPK/ERK kinase) and examined the effects of adenoviral over-expression of CCN2 on MMP1 protein levels. Figure 4c shows that UO126 prevented the CCN2-induced ERK1/2 activation and significantly inhibited the phosphorylation of Ets1 in response to CCN2 over-expression. Additionally, treatment of cells with the UO126 inhibitor completely abolished the CCN2-induced MMP1 up-regulation, suggesting ERK1/2/Ets1 as the downstream mediators of these effects. CCN2 overexpression was accompanied by c-Jun phosphorylation that was not abrogated by pretreatment with the ERK1/2 inhibitor.

In summary, these results suggest that up-regulation of MMP1 following Akt inhibition is mediated via a CCN2/ERK1/2/Ets1-dependent pathway.

Discussion

Wound healing is a complex process that requires the coordinated action of various molecules, among which TGF β , Akt, CCN2 and MMP1 have been shown to play important roles (1). Published data have shown that unbalanced expression or activity of these factors could interfere with the course of normal wound healing. Down-regulated Akt signalling and higher levels of MMP1 expression have been previously linked to chronic wounds (5,16–19), and opposite findings have been described for hypertrophic scars (4,21). However, a correlation between Akt and MMP1 levels in impaired wound healing has not been yet

established. In a previous study, we showed that blockade of Akt in human dermal fibroblasts has dual antifibrotic effects by both upregulating MMP1 gene expression and downregulating collagen deposition, thus mimicking the conditions of chronic wounds. Herein, we show for the first time that Akt blockade leads to up-regulation of CCN2 and that CCN2 is required for the up-regulation of MMP1 under these conditions. Additionally, we demonstrate that overexpression of CCN2 is sufficient to mimic the effects of Akt inhibition in human dermal fibroblasts with respect to MMP1 synthesis, and that the ERK1/2/Ets1 pathway is a critical mediator of these responses (Fig. 5).

The present study provides support for the role of CCN2 in the induction of MMP1 levels in human dermal fibroblasts. Using adenovirus mediated overexpression of CCN2 and recombinant human CCN2 we show that MMP1 protein levels are induced in a dose-dependent manner. Our data are consistent with a study by Chen et al. that showed similar effects on MMP1 after fibroblasts adhesion to CCN2 (8), thus providing additional support for a role of CCN2 in matrix remodeling. These observations are in contrast to the well established role of CCN2 as a profibrotic agent. However, the profibrotic role of CCN2 is only documented downstream of TGF β . In a study by Mori et al., CCN2 alone was not able to induce fibrosis when injected subcutaneously in mice while it caused sustained fibrosis in the presence of TGF β . Additionally, inhibition of CCN2 suppressed the TGF β induction of collagen, suggesting that it is required for the profibrotic effect of TGF β (29). With respect to wound healing, TGF β was found to be elevated with a peak on day 3 in a rat wound model, coinciding with the initial inflammatory phase, followed by a rapid decline to normal levels by day 9. Interestingly, the induction of CCN2 had a different pattern, with the highest levels accumulating at day 9 after injury, when the granulation tissue is formed (30). In our study CCN2 alone did not consistently induce type I collagen and treatment of cells with higher doses of either recombinant CCN2 or adenovirus over-expressing CCN2 resulted in a decrease in collagen protein levels. This suggests that depending on the cellular context and the presence of other factors CCN2 could have both profibrotic and antifibrotic functions. Studies regarding the dynamics of MMP1 production in various phases of wound healing show that this collagenase is secreted predominantly during the postacute stages, similar to CCN2 accumulation (31). Because the major inducers of MMP1 secretion in fibroblasts are TNF α and IL-1, which are mainly released from neutrophils during the initial inflammatory phase of wound healing, upregulated CCN2 in the latter stages could represent an important physiological inducer of MMP1.

Our experimental conditions of Akt blockade mimic the conditions of chronic, non healing wounds, which in our study have been linked to the upregulation of MMP1 levels and downregulation of collagen deposition. Because of the positive role of CCN2 in MMP1 gene expression, we examined the effect of Akt blockade on CCN2 synthesis. We show here using siRNA and pharmacological inhibitors that Akt is a negative regulator of CCN2 gene expression in human dermal fibroblasts. We also show that CCN2 is required for the induction of MMP1 gene expression following Akt blockade. These results suggest that CCN2 may have important functions in chronic wound healing. Although because of its known profibrotic effects CCN2 has been speculated to enhance wound healing, it is still unclear whether it plays a role in chronic wounds. CCN2 is released during wound healing from platelets and its levels in these cells are 20-folds higher than those of any other growth factors (32). However, in a controlled study, the use of topical autologous platelet lysate in chronic venous ulcers had no major difference in healing outcome when compared to placebo (33). Further studies are required to assess the levels of CCN2 in chronic wounds and to establish whether it plays a role in impaired healing.

The data in this study provides new insights into the mechanism of CCN2-induced MMP1 up-regulation. JNK and ERK1/2 members of the MAPKs family have been implicated in the

regulation of MMP1 gene expression in cultured fibroblasts. JNK activation results in rapid phosphorylation of the AP1 family member c-Jun, enhancing its transcriptional activity. ERK1/2 activates the Ets1 transcription factor that in turn cooperates with the AP-1 proteins c-Fos and c-Jun in inducing the MMP1 promoter (14,15). Here, we show that ERK1/2 and Ets1 are the downstream mediators of CCN2 effects on MMP1 gene expression. Treatment of cells with recombinant CCN2 or CCN2 overexpressing adenovirus resulted in phosphorylation of ERK, c-Jun and Ets1. Although the involvement of ERK1/2 and JNK in mediating CCN2 signalling has been described in chondrocytes (34), to our knowledge this report is the first to indicate that CCN2 can induce Ets1 phosphorylation. To assess whether the induction of ERK1/2/Ets1 could contribute to the CCN2-induced MMP1 up-regulation we measured the effects of the ERK1/2 pharmacological inhibitor UO126 in our experimental system. Treatment with UO126 resulted in complete abrogation of MMP1 induction and blockade of ERK1/2 and Ets1 phosphorylation following CCN2 overexpression, suggesting that this effect is mediated via ERK1/2/Ets1 activation. Consistent with the stimulatory role of ERK1/2 in the expression of CTGF and MMP1 genes, adenovirus-mediated transfer of dominant-negative Akt₁ enhanced the basal and TGF β -induced phosphorylation of ERK1/2 in a time-dependent manner.

Taken together our data underline the importance of a tight regulation of Akt, CCN2 and MMP1 in the process of normal wound healing. We propose a mechanism by which upregulated CCN2 in the postacute stages of tissue injury induces the expression of MMP1 that is required for efficient wound remodelling. Additionally, we hypothesize that the activation status of Akt could be an important regulator of the intensity of this process. Thus, low levels of Akt signalling could enhance the expression of CCN2 and trigger exaggerated MMP1 secretion via ERK1/2/Ets1 activation. Elevated MMP1 would in turn result in excessive matrix remodelling and dysregulation of the normal process of wound healing. Our results also suggest that Akt inhibitors could prove very useful in the treatment of hypertrophic scars. On the other hand, treatment with phosphatase and tensin homologue (PTEN) inhibitors (35,36) resulting in Akt activation could be beneficial for chronic, slow healing wounds. Thus, understanding the mechanism of coordinate regulation of MMP1, Akt and CCN2 could have important therapeutic implications in wound healing.

References

1. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev*. 2003; 83:835–870. [PubMed: 12843410]
2. Agarwal S, Achari C, Praveen D, Roy KR, Reddy GV, Reddanna P. Inhibition of 12-LOX and COX-2 reduces the proliferation of human epidermoid carcinoma cells (A431) by modulating the ERK and PI3K-Akt signalling pathways. *Exp Dermatol*. 2009; 18:939–946. [PubMed: 19558494]
3. Kim D, Chung J. Akt: versatile mediator of cell survival and beyond. *J Biochem Mol Biol*. 2002; 35:106–115. [PubMed: 16248975]
4. Aarabi S, Bhatt KA, Shi Y, et al. Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis. *FASEB J*. 2007; 21:3250–3261. [PubMed: 17504973]
5. Goren I, Muller E, Schiefelbein D, et al. Akt1 controls insulin-driven VEGF biosynthesis from keratinocytes: implications for normal and diabetes-impaired skin repair in mice. *J Invest Dermatol*. 2009; 129:752–764. [PubMed: 18668138]
6. Bujor AM, Pannu J, Bu S, Smith EA, Muise-Helmericks RC, Trojanowska M. Akt blockade downregulates collagen and upregulates MMP1 in human dermal fibroblasts. *J Invest Dermatol*. 2008; 128:1906–1914. [PubMed: 18323784]
7. de Winter P, Leoni P, Abraham D. Connective tissue growth factor: structure-function relationships of a mosaic, multifunctional protein. *Growth Factors*. 2008; 26:80–91. [PubMed: 18428027]

8. Chen CC, Chen N, Lau LF. The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *J Biol Chem*. 2001; 276:10443–10452. [PubMed: 11120741]
9. Daniels JT, Schultz GS, Blalock TD, et al. Mediation of transforming growth factor-beta(1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol*. 2003; 163:2043–2052. [PubMed: 14578203]
10. Kondo S, Kubota S, Shimo T, et al. Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. *Carcinogenesis*. 2002; 23:769–776. [PubMed: 12016149]
11. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol*. 2001; 17:463–516. [PubMed: 11687497]
12. Mauviel A. Cytokine regulation of metalloproteinase gene expression. *J Cell Biochem*. 1993; 53:288–295. [PubMed: 8300745]
13. Lee YM, Li WH, Kim YK, Kim KH, Chung JH. Heat-induced MMP-1 expression is mediated by TRPV1 through PKC α signaling in HaCaT cells. *Exp Dermatol*. 2008; 17:864–870. [PubMed: 18503554]
14. Reunanen N, Westermarck J, Hakkinen L, et al. Enhancement of fibroblast collagenase (matrix metalloproteinase-1) gene expression by ceramide is mediated by extracellular signal-regulated and stress-activated protein kinase pathways. *J Biol Chem*. 1998; 273:5137–5145. [PubMed: 9478967]
15. Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res*. 2002; 4:157–164. [PubMed: 12010565]
16. Barone EJ, Yager DR, Pozez AL, et al. Interleukin-1 α and collagenase activity are elevated in chronic wounds. *Plast Reconstr Surg*. 1998; 102:1023–1027. discussion 8–9. [PubMed: 9734419]
17. Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. *Diabetologia*. 2002; 45:1011–1016. [PubMed: 12136400]
18. Wall SJ, Bevan D, Thomas DW, Harding KG, Edwards DR, Murphy G. Differential expression of matrix metalloproteinases during impaired wound healing of the diabetes mouse. *J Invest Dermatol*. 2002; 119:91–98. [PubMed: 12164930]
19. Weckroth M, Vaheri A, Lauharanta J, Sorsa T, Kontinen YT. Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol*. 1996; 106:1119–1124. [PubMed: 8618050]
20. Dasu MR, Hawkins HK, Barrow RE, Xue H, Herndon DN. Gene expression profiles from hypertrophic scar fibroblasts before and after IL-6 stimulation. *J Pathol*. 2004; 202:476–485. [PubMed: 15095275]
21. Ghahary A, Shen YJ, Nedelec B, Wang R, Scott PG, Tredget EE. Collagenase production is lower in post-burn hypertrophic scar fibroblasts than in normal fibroblasts and is reduced by insulin-like growth factor-1. *J Invest Dermatol*. 1996; 106:476–481. [PubMed: 8648180]
22. Gore-Hyer E, Shegogue D, Markiewicz M, et al. TGF-beta and CTGF have over-lapping and distinct fibrogenic effects on human renal cells. *Am J Physiol Renal Physiol*. 2002; 283:F707–F716. [PubMed: 12217862]
23. Malmlof M, Roudier E, Hogberg J, Stenius U. MEK-ERK-mediated phosphorylation of Mdm2 at Ser-166 in hepatocytes. Mdm2 is activated in response to inhibited Akt signaling. *J Biol Chem*. 2007; 282:2288–2296. [PubMed: 17107963]
24. Rommel C, Clarke BA, Zimmermann S, et al. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science*. 1999; 286:1738–1741. [PubMed: 10576741]
25. Wang B, Yang Y, Friedman PA. Na/H exchange regulatory factor 1, a novel AKT-associating protein, regulates extracellular signal-regulated kinase signaling through a B-Raf-mediated pathway. *Mol Biol Cell*. 2008; 19:1637–1645. [PubMed: 18272783]
26. Zhang D, Bar-Eli M, Meloche S, Brodt P. Dual regulation of MMP-2 expression by the type 1 insulin-like growth factor receptor: the phosphatidylinositol 3-kinase/Akt and Raf/ERK pathways transmit opposing signals. *J Biol Chem*. 2004; 279:19683–19690. [PubMed: 14993222]

27. Yosimichi G, Nakanishi T, Nishida T, Hattori T, Takano-Yamamoto T, Takigawa M. CTGF/Hcs24 induces chondrocyte differentiation through a p38 mitogen-activated protein kinase (p38MAPK), and proliferation through a p44/42 MAPK/extracellular-signal regulated kinase (ERK). *Eur J Biochem.* 2001; 268:6058–6065. [PubMed: 11732999]
28. Bu S, Yamanaka M, Pei H, et al. Dihydrosphingosine 1-phosphate stimulates MMP1 gene expression via activation of ERK1/2-Ets1 pathway in human fibroblasts. *FASEB J.* 2006; 20:184–186. [PubMed: 16278291]
29. Mori T, Kawara S, Shinozaki M, et al. Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: A mouse fibrosis model. *J Cell Physiol.* 1999; 181:153–159. [PubMed: 10457363]
30. Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Biol Cell.* 1993; 4:637–645. [PubMed: 8374172]
31. Arumugam S, Jang YC, Chen-Jensen C, Gibran NS, Isik FF. Temporal activity of plasminogen activators and matrix metalloproteinases during cutaneous wound repair. *Surgery.* 1999; 125:587–593. [PubMed: 10372023]
32. Kubota S, Kawata K, Yanagita T, Doi H, Kitoh T, Takigawa M. Abundant retention and release of connective tissue growth factor (CTGF/CCN2) by platelets. *J Biochem.* 2004; 136:279–282. [PubMed: 15598883]
33. Stacey MC, Mata SD, Trengove NJ, Mather CA. Randomised double-blind placebo controlled trial of topical autologous platelet lysate in venous ulcer healing. *Eur J Vasc Endovasc Surg.* 2000; 20:296–301. [PubMed: 10986030]
34. Yosimichi G, Kubota S, Nishida T, et al. Roles of PKC, PI3K and JNK in multiple transduction of CCN2/CTGF signals in chondrocytes. *Bone.* 2006; 38:853–863. [PubMed: 16431170]
35. Schmid AC, Byrne RD, Vilar R, Woscholski R. Bisperoxovanadium compounds are potent PTEN inhibitors. *FEBS Lett.* 2004; 566:35–38. [PubMed: 15147864]
36. Zhao M. PTEN: a promising pharmacological target to enhance epithelial wound healing. *Br J Pharmacol.* 2007; 152:1141–1144. PMCID: 2189987. [PubMed: 17922021]

Figure 1.

CCN2 is a positive regulator of matrix metalloproteinase 1 (MMP1). Equal numbers of human dermal fibroblasts were plated and grown to confluence then serum-starved overnight. Cells were then treated with either CCN2 over-expressing or control (G0) adenovirus in (a) and with recombinant human CCN2 in (b). Western blot from conditioned media collected 72 h latter shows that compared to control, cells treated with CCN2 secreted more MMP1 and less collagen type I, in a dose-dependent manner. Bar graphs in (b) represent means $\pm$ SEM of three independent experiments in two different cell lines, with * P 0.05; ** P 0.01.

Figure 2.

The effects of Akt inhibition on basal and TGF β -induced CCN2 levels. (a) Dose-dependent increase in CCN2 with Akt inhibitor VIII. Dermal fibroblasts were grown to confluence and serum-starved overnight then treated with either Akt inhibitor VIII (2.5, 5, 10, 15 and 20 μ M) or DMSO for 2 days. Cells were scraped and the levels of CCN2 were analysed by Western blot. (b) Time-dependent up-regulation of CCN2 mRNA levels after Akt inhibition. Dermal fibroblasts were treated with 20 μ M of Akt inhibitor VIII for the indicated times and then the mRNA levels were analysed by qRT-PCR. mRNA values were normalized relative to control, DMSO-treated cells (arbitrarily set as 1) and means $\pm$ SEM of four independent experiments in three different cell lines are shown. * P < 0.05; ** P < 0.01 versus control, vehicle-treated cells. (c) Representative Western blot showing the effect of Akt 1, 2 and 3 siRNA on CCN2 protein levels. The levels of total Akt, CCN2 and β -actin were analysed by Western blot. Representative data from three different experiments are shown with quantitative analysis of the results at right: the bar graph shows means $\pm$ SEM with values normalized to control, scrambled siRNA-treated cells. * P < 0.05; ** P < 0.01. (d) Equal numbers of dermal fibroblasts were grown to confluence and serum-starved overnight then treated with increasing doses of Akt inhibitor VIII for 72 h. A representative Western blot of conditioned media showing the levels of CCN2 and matrix metalloproteinase 1 (MMP1) is shown. The experiments were repeated at least three times in two different cell lines. (e) Confluent dermal fibroblasts were serum-starved overnight and pretreated with 20 μ M of Akt inhibitor VIII for 1 h, followed by TGF β stimulation for 72 h. Cells were collected and the

levels of CCN2 and MMP1 were analysed by Western blot or qRT-PCR for MMP1 in (f). - actin was used as a control. Experiments were repeated at least three times in two different cell lines, and a representative gel is shown. * $P < 0.05$; ** $P < 0.01$.

Figure 3.

Increased CCN2 in response to Akt blockade contributes to matrix metalloproteinase 1 (MMP1) up-regulation. (a) Effects of adenoviral-mediated CCN2 overexpression and Akt inhibitor VIII on MMP1 levels. Equal numbers of dermal fibroblasts were grown to confluence, serum-starved overnight and then treated with 20 μ M of Akt inhibitor VIII or transduced with adenovirus overexpressing CCN2 for 72 h. DMSO and G0 adenovirus were used as control. Western blot analysis of conditioned media shows the increase in MMP1 secretion of cells with different levels of CCN2 up-regulation. A representative blot is presented and the bar graph shows means $\pm$ SEM of three independent experiments with values normalized to control, vehicle-treated cells, * P < 0.05, ** P < 0.01. (b) Effects of siRNA-mediated CCN2 blockade on Akt inhibitor VIII-mediated MMP1 up-regulation. Equal numbers of dermal fibroblasts were plated and treated with adenovirus overexpressing CCN2siRNA in 10% fetal bovine serum. Twenty-four hours later media was changed to serum free media and cells were treated with 2.5 ng/ml of TGF or 20 μ M of Akt inhibitor VIII. Conditioned media collected after 72 h was analysed by Western blot for secreted CCN2 and MMP1. Representative blots are shown with quantitative analysis of three different experiments in three different cell lines on the right panel. The values were normalized to control, vehicle-treated cells, * P < 0.05, ** P < 0.01.

Figure 4.

CCN2 induces matrix metalloproteinase 1 (MMP1) gene expression via an ERK1/2/Ets1-dependent mechanism. (a) Adenovirus-mediated transfer of dominant-negative Akt₁ enhances the basal and TGF β -induced phosphorylation of Erk1/2 in human dermal fibroblasts in a time-dependent manner. Dermal fibroblasts were transduced with either AdDnAkt₁ or control virus (LacZ) at MOI 50, as described. Cells were treated after 48 h with 1 ng/ml of TGF β for the indicated time points and then collected. The levels of phospho-ERK1/2 and total ERK1/2 were analysed by Western blot. Experiments were repeated twice in two different cell lines. (b) Time-dependent induction of Ets1 and ERK1/2 phosphorylation by CCN2. Cells were treated with recombinant human CCN2 for the indicated time points and the levels of P-Ets1, Ets1, P-ERK1/2 and ERK1/2 were analysed by Western blot. Experiments were repeated twice in two different cell lines. (c) Effects of UO126 on cells overexpressing CCN2. Dermal fibroblasts were pretreated with UO126 for an hour and then transduced with either AdCCN2 or control virus (LacZ) as described. The levels of CCN2, MMP1, P-Ets1, P-ERK1/2 and P-c-Jun were analysed by Western blot 48 h after transduction. β -actin was used as a control for equal loading. Representative gels are shown. Experiments were repeated twice in two different cell lines.

Figure 5.

The effects of Akt activation status on matrix metalloproteinase 1 (MMP1) gene expression. In the presence of pharmacological inhibitors, signalling through Akt is blocked resulting in an increase in CCN2 protein levels, which in turn activates ERK1/2/Ets1 signalling leading to transcriptional activation of the MMP1 promoter. Conversely, activated Akt downstream of TGF or other stimuli may be a negative regulator of MMP1 gene expression via additional unknown mechanisms.