CCN1 contributes to skin connective tissue aging by inducing age-associated secretory phenotype in human skin dermal fibroblasts

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Received: 23 May 2011 / Accepted: 21 June 2011 / Published online: 1 July 2011
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Abstract Dermal connective tissue collagen is the major structural protein in skin. Fibroblasts within the dermis are largely responsible for collagen production and turnover. We have previously reported that dermal fibroblasts, in aged human skin in vivo, express elevated levels of CCN1, and that CCN1 negatively regulates collagen homeostasis by suppressing collagen synthesis and increasing collagen degradation (Quan et al. *Am J Pathol* 169:482–90, 2006, *J Invest Dermatol* 130:1697–706, 2010). In further investigations of CCN1 actions, we find that CCN1 alters collagen homeostasis by promoting expression of specific secreted proteins, which include matrix metalloproteinases and proinflammatory cytokines. We also find that CCN1-induced secretory proteins are elevated in aged human skin in vivo. We propose that CCN1 induces an “Age-Associated Secretory Phenotype”, in dermal fibroblasts, which mediates collagen reduction and fragmentation in aged human skin.

Keywords CCN1 · Age-associated secretory phenotype · Skin connective tissue aging

Abbreviations

CCN1	cysteine-rich protein 61
CCN family	cysteine-rich protein 61, connective tissue growth factor, nephroblastoma overexpressed
AFM	atomic force microscopy
MMP	matrix metalloproteinases

ECM	extracellular matrix
PCR	polymerase chain reaction
IL	interleukin
NF- κ B	nuclear factor kappa B

Introduction

Collagen is the major extracellular matrix (ECM) component of the skin, and provides structural and functional support. Alterations of skin connective tissue collagen are prominent features of aged human skin (Fisher et al. 2002, 2009; Varani et al. 2006, 2000). These alterations largely result from aberrant collagen homeostasis in dermal fibroblasts, the primary cells responsible for the synthesis and degradation of connective tissue collagen. However, it remains unclear the underlying molecular mechanisms that are responsible for age-related aberrant collagen homeostasis in human skin. Here we report that elevated CCN1 in aged human skin functions as a novel mediator of skin connective tissue aging by up-regulating production of specific secreted proteins in human dermal fibroblasts. We term this expression pattern “Age-Associated Secretory Phenotype”.

CCN1 is member of the CCN family of proteins, which at present consists of six distinct members; cysteine-rich protein 61 (CCN1), connective tissue growth factor (CCN2), nephroblastoma overexpressed (CCN3), Wnt-induced secreted protein-1 (CCN4), Wnt-induced secreted protein-2 (CCN5), and Wnt-induced secreted protein-3 (CCN6) (Chen and Lau 2009; Lau and Lam 1999b; Leask and Abraham 2006; Perbal 2004). The CCN family members are matricellular proteins, which are secreted and associate with the ECM. CCN family proteins have

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been shown to exhibit diverse cellular functions, such as regulation of cell adhesion, proliferation, migration, chemotaxis, apoptosis, motility, and wound healing ECM remodeling (Lau and Lam 1999a). We have previously reported that CCN1 is predominantly expressed in human dermal fibroblasts, and elevated CCN1 modulates type I collagen homeostasis by down-regulating its production and promoting its degradation, in cultured dermal fibroblasts (Quan et al. 2006, 2010). In further investigations of CCN1 actions, we found that elevated CCN1 levels cause dermal fibroblasts to acquire an age-associated secretory phenotype, and thus contribute to human skin connective tissue aging.

Methods

Procurement of human skin in vivo samples Sun-protected buttock human skin punch biopsies were obtained from healthy adult human volunteers, as previously described (Fisher et al. 1998, 1997). Young and aged subjects were grouped according to age: 21–30 years for young group and 80+ years for aged group. Skin samples were 4 mm in diameter, full thickness skin. The study involving human subjects was approved by the institutional review board at the University of Michigan, and all subjects provided written informed consent before entering the study.

Atomic force microscopy (AFM) imaging Human skin biopsies were embedded in OCT and cryo-sections (10 μ m) were mounted on microscope cover glass (1.2 mm diameter, Fisher Scientific Co., Pittsburgh, PA). These samples were allowed to air dry for at least 24 h before AFM image analysis. AFM images were acquired using a Multimode Nanoscope IIIa AFM system (Veeco Instrument Inc., Santa Barbara, CA), as previously described (Quan et al. 2011). Briefly, the scan positions of the skin dermis were determined by light optical image. AFM images were obtained with tapping mode using a silicon etched cantilever (NSC15/AIBS, MikroMasch, San Jose, CA) with a full tip cone angle $\sim 40^\circ$ and tip radius of curvature ~ 10 nm. AFM images were acquired at a scan rate of 1.0 Hz, 256 $\times$ 256 pixel resolution, integral and proportional gain settings of 0.4 and 0.6 respectively. The image quality was optimized by dynamically lowering the scan rate and setpoint, and increasing the gains and drive amplitude. AFM images were obtained from Electron Microbeam Analysis Laboratory (EMAL), University of Michigan College of Engineering, and analyzed using Nanoscope Analysis software (Veeco Instrument Inc.).

Cell culture and transfection Adult human primary dermal fibroblasts were prepared from punch biopsies of adult

normal buttock skin (aged 21–55 years), as described previously (Fisher et al. 1991). Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100 units/ml), streptomycin (100 μ g/ml) in a humidified incubator with 5% CO₂ at 37°C. Cells were plated at 70–80% confluence, and used one day after plating. Cells were utilized between passages 3 and 10. Independent replicates of studies, indicated by N number in figure legends, were performed with cells from different individuals. For transfection, human CCN1 cDNA expression vector was cloned into pCDNA3.1 expression vector (Invitrogen, Carlsbad, CA), as described previously (Quan et al. 2006). Human skin dermal fibroblasts were transiently transfected by electroporation (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's protocol. After 48 h of transfection, total RNA was extracted and target gene mRNA levels were determined by real-time RT-PCR, as described below.

RNA isolation and quantitative real-time PCR Total RNA was isolated from human skin punch biopsy or cultured human skin dermal fibroblasts using a commercial kit (RNeasy mini kit, Qiagen, Chatsworth, CA) according to the manufacturer's protocol. PCR template was prepared by reverse transcription using Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA). PCR was performed in duplicate with 2 μ l of cDNA for all genes of interest using TaqMan Universal PCR Master Mix kit (Applied Biosystems) and a 7300 sequence detector system (Applied Biosystems). PCR procedures were performed with a robotic workstation (Biomek 2000; Beckman Coulter, Inc., Hialeah, FL) to ensure accuracy and reproducibility. PCR primers and probes were from Applied Biosystems custom oligonucleotide synthesis service. Primers and FAM-labeled probes for real-time PCR were as follows: CCN1 forward primer, 5'-TCAAA-GACCTGTGGAAGTGGTATC-3', reverse primer, 5'-CACAAATCCGGGTTTCTTTCA-3', and probe, 5'-CAAT GACAACCCTGAGTGCCGCCT-3'; Type III procollagen, forward primer, 5'-TCTTGGTCAGTCCTATGCGGATA-3', reverse primer, 5'-CATCGCAGAGAACGGATCCT-3', and probe, 5'-CCAGAACCATGCCAAATATGTGTCTGTGACT-3'; IL-1 β , forward primer, 5'-TTCTTCGACACATGGGA TAACG-3', reverse primer, 5'-TCCCGGAGCGTG CAGTTCA-3', and probe, 5'-TAACGAGGCTTATGTGCAC GATGCACCTGTACGATCA-3'; IL-6, forward primer, 5'-GATGGATGCTTCCAATCTGGAT-3', reverse primer, 5'-AGGTACTCTAGGTATACCTCAAACCTCAA-3'. MMPs and 36B4 primers and probes were described previously (Quan et al. 2002, 2004, 2010). Multiplex PCR reactions contained primers and probes for target gene and 36B4, a ribosomal protein used as an internal normalization control for quantitation. PCR amplification condition: 94°C, 30 s;

54–60°C, 1 min; 68°C, 1 min for 35 cycles, 72°C, 10 min. Type III procollagen and MMPs mRNA levels in human skin in vivo were quantified based on standard curves for each individual gene.

Statistical analysis Data are expressed as mean $\pm$ SEM. Student's *t*-test was used to evaluate the statistical differences between the groups. All *p* values are two-tailed, and less than 0.05 was considered statistically significant.

Results

Alterations of dermal connective tissue collagen are prominent features of chronologically aged human skin dermis Prominent alterations in the structural organization of collagen fibrils are seen in the dermis of chronologically aged skin. Figure 1 shows nanoscale three-dimensional topographical images of skin connective tissue collagen imaged by atomic force microscopy (AFM). AFM images reveal that collagen fibrils in young skin dermis are intact, abundant, tightly packed, and well-organized (Fig. 1a). In contrast, collagen fibrils in aged skin dermis are fragmented, sparse and disorganized (Fig. 1b). Quantitative analysis of AFM data further indicates that the average roughness (a measure of fibril organization) of dermal collagen fibrils in aged skin is 2.7 times greater than in young skin (52.5 nm vs 19.1 nm). These results demonstrate that alterations of dermal collagen integrity and structure are prominent features of aged human skin dermis.

CCN1 down-regulates type III procollagen in human skin dermal fibroblasts, as observed in human skin in vivo The bulk of skin is comprised of the connective tissue dermis, in

which type I and type III collagen are the major components. In human skin, dermal fibroblasts are responsible for collagen production. We have previously reported that aged dermal fibroblasts, in human skin in vivo, express elevated levels of CCN1, which inhibits type I procollagen expression (Quan et al. 2006). In further investigation of CCN1 actions, we found that over-expression of CCN1 not only reduces type I procollagen, but also reduces type III collagen (Fig. 2a), as observed in aged human skin in vivo (Fig. 2b). These data suggest that elevated CCN1 down-regulates both type I and type III collagen in aged human skin.

CCN1 up-regulates MMP in human skin dermal fibroblasts, as observed in human skin in vivo MMPs catalyze fragmentation of the dermal collagenous extracellular matrix. We have previously reported that elevated CCN1 up-regulates interstitial collagenase (MMP-1), which initiates cleavage of collagen fibrils, in human skin dermal fibroblasts. Further investigation of CCN1 actions show that over-expression of CCN1 not only induces MMP-1, but also induces stromelysin-1 (MMP-3), and 92 kDa gelatinase (MMP-9) (Fig. 3a), as observed in aged human skin in vivo (Fig. 3b). These data suggest that CCN1 up-regulates multiple MMPs, which may promote collagen degradation in aged human skin.

CCN1 up-regulates IL-1 β and IL-6 in human skin dermal fibroblasts, as observed in human skin in vivo It has been proposed that low grade chronic elevation of proinflammatory cytokines can be a driving force for the aging process (Franceschi et al. 2007; Goto 2008; Maggio et al. 2006; Singh and Newman 2011). We found that over-expression of CCN1 in human skin dermal fibroblasts resulted in significant up-regulation of IL-1 β and IL-6, two important proinflammatory cytokines (Fig. 4a). Interestingly, IL-1 β

Fig. 1 Alterations of dermal connective tissue collagen are characteristic features of chronologically aged human skin. Nanoscale three-dimensional human skin connective tissue collagen fibrils were analyzed by atomic force microscopy (AFM). Representative AFM images of young (a) and aged (b) human skin dermis. The white

arrows indicate intact collagen fibrils of young skin dermis (a), and black arrows indicate damaged collagen fibrils in aged human skin dermis (b). AFM data are representative of five subjects from each age group

Fig. 2 CCN1 down-regulates type III procollagen in human skin dermal fibroblasts, as observed in human skin in vivo. **a** Human skin fibroblasts were transfected with control vector (pcDNA3.1) or CCN1 expression vector. Cells were harvested 48 h after transfection. Total RNA was extracted and type III procollagen mRNA levels were quantified by real-time RT-PCR and normalized to 36B4 mRNA (housekeeping gene, internal control). Results are expressed as means $\pm$ SEM, $N=6$, $*p<0.05$. **b** Young and aged human buttock skin was obtained and total RNA was extracted. Type III procollagen mRNA levels were quantified using real-time RT-PCR. Data were normalized to 36B4 mRNA (housekeeping gene, internal control) and expressed as copy numbers, based on standard curves. Results are expressed as means $\pm$ SEM, $N=7$, $*p<0.05$

(Fig. 4b) and IL-6 (Fig. 4c) are also up-regulated in aged human skin in vivo. These data suggest that CCN1 up-regulates IL-1 β and IL-6, which may contribute to the proinflammatory cytokine-mediated aging process in human skin.

CCN1 contributes to dermal connective tissue aging by inducing age-associated secretory phenotype in human skin dermal fibroblasts We have previously reported that elevated CCN1 modulates collagen homeostasis in human skin fibroblasts, by down-regulating type I procollagen and up-regulating MMP-1 (Quan et al. 2006, 2010). Based on our previous and current data, we propose that elevated CCN1 contributes to skin connective tissue aging by the abnormal production of specific secreted proteins, which we term “Age-Associated Secretory Phenotype” (Fig. 5). The characteristic features of CCN1-induced age-associated secretory phenotype are: 1) down-regulation of type I and type III collagen, which contributes to thin skin dermis; 2) up-regulation of MMPs (MMP-1, MMP-3, MMP-9), which contributes to fragmentation of the dermal ECM; and 3) up-regulation of proinflammatory cytokines (IL-1 β and IL-6), which promotes tissue damage. We propose that elevated CCN1 in aged human skin functions as a key mediator of

Fig. 3 CCN1 up-regulates MMP-3 and MMP-9 in human skin dermal fibroblasts, as observed in human skin in vivo. **a** Human skin fibroblasts were transfected with control vector (pcDNA3.1) or CCN1 expression vector. Cells were harvested 48 h after transfection. Total RNA was extracted and MMP-3 and MMP-9 mRNA levels were quantified by real-time RT-PCR and normalized to 36B4 mRNA (housekeeping gene, internal control). Results are expressed as means $\pm$ SEM, $N=4$, $*p<0.05$. **b** Young and aged human buttock skin was obtained and total RNA was extracted. MMP-3 and MMP-9 mRNA levels were quantified using real-time RT-PCR. The data were normalized to 36B4 mRNA (housekeeping gene, internal control) and expressed as copy numbers based on standard curves. Results are expressed as means $\pm$ SEM, $N=6$, $*p<0.05$

age-dependent deterioration of dermal connective tissue, by inducing age-associated secretory phenotype in human skin dermal fibroblasts.

Discussion

Emerging evidence indicates that CCN proteins may represent a new class of modulators of inflammation (Jun and Lau 2010b; Kular et al. 2011). Consistent with this notion, CCN1 activates a proinflammatory genetic (Bai et al. 2010). Furthermore, evidence indicates a potential role of CCN1 in chronic inflammatory diseases such as atherosclerosis, rheumatoid arthritis, inflammatory kidney diseases and neuroinflammatory diseases (Bai et al. 2010; Kular et al. 2011). It has been proposed that low grade chronic elevation of proinflammatory mediators can be a driving force for aging progression (Franceschi et al. 2007; Goto 2008; Maggio et al. 2006; Singh and Newman 2011). Central to this concept is that healthy aging is not an

Fig. 4 CCN1 up-regulates IL-1 β and IL-6 mRNA in human skin dermal fibroblasts, as observed in human skin in vivo. **a** Human skin fibroblasts were transfected with control vector (pcDNA3.1) or CCN1 expression vector. Cells were harvested 48 h after transfection. Total RNA was extracted and IL-1 β and IL-6 mRNA levels were quantified by real-time RT-PCR and normalized to 36B4 mRNA (housekeeping gene, internal control). Results are expressed as means $\pm$ SEM, $N=3$, $*p<0.05$. **(b and c)** Young and aged human buttock skin was obtained and total RNA was extracted. IL-1 β **(b)** and IL-6 **(c)** mRNA levels were quantified using real-time RT-PCR. The data were normalized to the 36B4 mRNA (housekeeping gene, internal control), and expressed as copy numbers, based on standard curves. Results are expressed as means $\pm$ SEM, IL-1 β , $N=6$; IL-6, $N=12$, $*p<0.05$

Fig. 5 Proposed model for the role of CCN1-induced age-associated secretory phenotype in human skin aging. CCN1 is elevated in dermal fibroblasts in aged human skin. Elevated CCN1 promotes an age-associated secretory phenotype, characterized by reduced collagen production, and elevated collagen-degrading MMPs as well as proinflammatory cytokines. These alterations conspire to impair dermal connective tissue structure and function, thereby leading to thin and fragile skin in the aged

inflammatory disease, but rather sub-clinical inflammation contributes to the gradual decline of organ function, which occurs during the aging process. For example, IL-6, which is an age-associated secretory phenotype protein, is increased in the aged and has been suggested to be a marker of health status in the elderly (Ershler et al. 1993; Maggio et al. 2006). Interestingly, our data indicate that IL-6 is elevated in aged skin and induced by CCN1 in human skin dermal fibroblasts. Although we and others agree that aged skin does not display overt inflammation, the functional role of subtle constitutive increases of cytokine levels in human skin connective tissue aging has not been investigated. It is conceivable that low level chronic elevation of IL-1 β and IL-6 may contribute to sustained upregulation of MMPs and collagen loss since these cytokines have been shown to negatively regulate collagen homeostasis (Bauge et al. 2007; Lencel and Magne 2011; Maggio et al. 2006; Wlaschek et al. 1994). Therefore, the possibility that CCN1-inducible cytokines contribute to human skin connective tissue aging deserves further investigation.

Understanding molecular mechanisms by which CCN1 induces age-associated secretory phenotype in human skin dermal fibroblasts requires further study. It has been shown that CCN1 functions primarily through interactions with integrins in a cell-type, function-specific manner (Chen and Lau 2009; Lau and Lam 1999b; Leu et al. 2002; Miranti and Brugge 2002; Monnier et al. 2008; Schober et al. 2003; Stupack and Chersesh 2002). CCN1 has been reported to interact with integrins to increase intracellular levels of reactive oxygen species (ROS), which function as important effectors of CCN1 actions (Jun and Lau 2010a; Jun and Lau 2010b). The ability of CCN1 to induce age-associated secretory phenotype in dermal fibroblasts may be mediated by integrin-coupled generation of ROS. It is well-documented that the integrin pathway and ROS lead to stimulation of MAPK and NF- κ B signaling (Cabodi et al. 2010; Stupack and Chersesh 2002; Svineng et al. 2008), which up-regulate MMPs and proinflammatory cytokines, including IL-1 β and IL-6 (Fisher et al. 1996; Fisher and Voorhees 1998; Maggio et al. 2006; Vincenti and Brinckerhoff 2002; Westermarck et al. 2001). Therefore, it is conceivable that elevated CCN1 upregulates MMPs and cytokines through activation of integrin and/or ROS-mediated activation of MAPK and NF- κ B signaling in aged human skin dermal fibroblasts. It is also conceivable that elevated IL-1 β and IL-6 drives not only upregulation of MMPs but also downregulation of collagen synthesis, since these cytokines have been shown to negatively regulate collagen homeostasis (Bauge et al. 2007; Maggio et al. 2006).

We have previously reported that CCN1 significantly impairs TGF- β signaling, as a result of down-regulation of TGF- β type II receptor (Quan et al. 2006). TGF- β

signaling plays a critical role in regulating collagen homeostasis by modulating both collagen biosynthesis and degradation (Chang and Goldberg 1995; Chen et al. 2000; Hall et al. 2003; Ignatz and Massague 1986; Inagaki et al. 1994; White et al. 2000). Interestingly, IL-1 β has been shown to impair TGF- β signaling by down-regulation of TGF- β type II receptor, thereby promoting development of degenerative and inflammatory diseases in osteoarthritis (Bauge et al. 2007). Therefore, it is possible that elevated CCN1 impairs TGF- β signaling through upregulation of IL-1 β , and thereby contributes to aberrant collagen homeostasis in aged human skin dermal fibroblasts. Indeed, we have previously reported that TGF- β pathway is impaired in aged human skin in vivo, largely due to down-regulation of TGF- β type II receptor (Quan et al. 2004; Quan et al. 2006).

In summary, here we report that elevated CCN1 causes dermal fibroblasts to acquire an age-associated secretory phenotype, and thus contributes to human skin connective tissue aging. Age-associated secretory phenotype brings about characteristic features of aging skin, through creating a tissue environment that promotes reduced production and increased degradation of collagen. With the passage of time, the accumulated impact of age-associated secretory phenotype-mediated cellular and tissue environment alterations leads to loss of function and integrity that are characteristic features of skin connective tissue aging.

Acknowledgments We thank Suzan Rehbine for the procurement of tissue specimens, and Diane Fiolek for graphic and administrative assistance. This work was supported by the National Institute of Health (ES014697 and ES014697 30S1 to T Quan; AG019364 and AG025186 to G Fisher).

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