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Minireview

Oxidative stress and CCN1 protein in human skin connective tissue aging

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Abstract: Reactive oxygen species (ROS) is an important pathogenic factor involved in human aging. Human skin is a primary target of oxidative stress from ROS generated from both extrinsic and intrinsic sources, like ultraviolet irradiation (UV) and endogenous oxidative metabolism. Oxidative stress causes the alterations of collagen-rich extracellular matrix (ECM), the hallmark of skin connective tissue aging. Age-related alteration of dermal collagenous ECM impairs skin structural integrity and creates a tissue microenvironment that promotes age-related skin diseases, such as poor wound healing and skin cancer. Here, we review recent advances in our understanding of oxidative stress and CCN1 protein (first member of CCN family proteins), a critical mediator of oxidative stress-induced skin connective tissue aging.

Keywords: ROS; oxidative stress; CCN1; skin aging; fibroblasts; ECM; collagen

Abbreviations: ROS, reactive oxygen species; CCN1, first member of CCN family proteins: AASP, age-associated secretory phenotype; AADM, age-associated dermal microenvironment; ECM, extracellular matrix; UV, ultraviolet, FN, fibronectin

1. Introduction

Human skin is the largest and the heaviest organ of the human body. The primary function of the skin is to provide a protective barrier to harmful environmental factors, such as solar UV, pathogens, physical and chemical insults. The human skin is composed of three layers: outermost epidermal layer, the vascular connective tissue dermis below the epidermis, and the hypodermis (the deepest portion of the skin). The epidermis is primarily composed of keratinocytes, which produce a class of proteins known as keratins that are a major component of the protective horny layer. The

subcutaneous hypodermis consists of fat cells dispersed throughout the connective tissue framework. The dermis is less cellular than the epidermis and is composed predominantly of ECM proteins, also known as the core matrisome [1,2], such as collagen, elastin, fibronectin, and proteoglycans. The dermis intimately interacts with the epidermis and subcutaneous fat to provide structural and mechanical support of the skin. Among many core matrisome proteins, collagen represents by far the most abundant protein that constitutes the bulk (90% dry weight) of skin [3]. In skin, dermal fibroblasts produce and organize the collagen-rich matrisome proteins, and thus maintains skin connective tissue homeostasis.

A large body of evidence indicates that damage to cellular constituents by reactive oxygen species (ROS) is a major driving force for the aging process [4,5]. It is proposed that ROS oxidize cellular constituents such as proteins, nucleic acids, and lipids. Accumulation of oxidative cellular damage, which occurs during the passage of time, impairs cellular function to yield the aged phenotype. Age-related increase of oxidative damage has been reported in a variety of human and animal tissues and involved in a number of human diseases [5,6]. Human skin is exposed to reactive oxygen species (ROS) generated from both environmental sources like ultraviolet irradiation (UV) (photoaging) [7] and endogenous oxidative metabolism (natural aging) [8]. These oxidative stresses causes alterations of dermal collagen-rich matrisome proteins, which impairs skin structural and mechanical integrity and creates a tissue microenvironment that promotes age-related skin diseases, such as poor wound healing [9,10] and skin cancer [11-13]. Therefore, oxidative stress is an important pathogenic factor involved in human skin aging process, as described below.

2. Oxidative stress and human skin aging

Aging skin is recognizable by fine and coarse wrinkles, skin laxity, coarseness, uneven pigmentation, and brown spots [14-16]. Based on its causes, skin aging can be classified into two types: natural aging, also known as intrinsic aging, and photoaging, also known as extrinsic aging caused by ultraviolet light from the sun [17-19]. Natural aging refers to those changes observed in all individuals resulting from the passage of time, whereas photoaging refers to those changes attributable to habitual sun exposure. Both processes are cumulative and therefore photoaging is superimposed on intrinsic aging. Therefore, the alterations seen in aged skin are a combination of intrinsic and extrinsic aging. The most clinically noticeable age-related changes occur on face, neck, forearm, and lower leg skin [20]. These areas undergo a combination of natural aging and photoaging and age-related skin diseases occur most often in these areas. As skin changes are among the most visible signs of aging, skin is central in the social and visual experience. Skin appearance has a significant emotional and psychological impact on our life quality. In clinically, aged skin has significant pathological impact on many age-related skin diseases.

Histological and ultrastructural studies have revealed skin undergoes remarkable morphologic changes with aging, which are primarily characterized by thinner dermis with reduced numbers of dermal fibroblasts [14,15]. These age-related thinning of the dermis is the major driving force for the most prominent clinical features of aged skin. It is well documented that age-related thinning of the dermis significantly impairs skin function such as increased fragility [21,22], impaired vasculature support [23-25], poor wound healing [25,26], and a tissue microenvironment that promotes cancer [13,27-29].

Oxidative stress is an important pathogenic factor for human aging [4,5]. Human skin is

exposed to ROS generated from both environmental sources like UV and endogenous oxidative metabolism. UV irradiation from the sun is a well-recognized, potent environmental insult capable of damaging skin tissue [7,30]. ROS generated by UV irradiation functions a primary mediator of human skin photoaging [31,32]. In aged skin, the levels of protein carbonyls, a well-established biomarker of oxidative damage, are significantly elevated in dermis of aged individuals, compared to young skin dermis [8]. There are two major sources of ROS in human skin; NADPH oxidase (NOX) in cell membranes [7,8], and mitochondria [33]. In human skin, mitochondria are the significant source of ROS generation. As ROS generation is significantly elevated in aged human skin, oxidative stress not only inhibits the production of collagen [34,35], but also stimulating collagen breakdown [8,35], and thus contribute to human skin aging, as described below.

3. CCN1 functions a critical mediator of oxidative stress-induced skin connective tissue aging

CCN1, also named cysteine-rich angiogenic inducer 61 (CYR61), is a first member of the CCN family of secreted proteins [36,37]. The CCN family comprises six distinct members: cysteine-rich protein 61 (CYR61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (NOV), Wnt-inducted secreted protein-1 (WISP1), Wnt-inducted secreted protein-2 (WISP2), and Wnt-inducted secreted protein-3 (WISP3) [38-40]. By international consensus, these genes have now been renamed CCN1-6 [41]. The CCN acronym is taken from the first three members described: CYR61, CTGF, and NOV [41]. All CCN proteins are secreted, ECM-associated matricellualr proteins. CCN family proteins are involved in a variety of cellular functions such as regulation of cell adhesion, proliferation, migration, chemotaxis, apoptosis, motility, and ECM remodeling in wound healing [36,42,43]. In cell culture models, CCN1 recombinant protein regulates cell adhesion, cell migration, cell-matrix interactions and the synthesis of extracellular matrix [44-46]. CCN1-deficiency in mice is embryonic lethal primarily due to failure of ECM remodeling and homeostasis [47]. Full-length CCN1precursor is 381 amino acids in length. CCN1, like other CCN family proteins, consists of four conserved structural/functional domains (Figure 1) [48]. These domains have sequence homologies to 1) insulin-like growth factor binding proteins (IGFBP), 2) Von Willebrand factor type C repeat (VWC), 3) thrombospondin type I repeat (TSR), and 4) C-terminal cysteine knots (CT). These four distinct structural domains are separated by proteases-sensitive residues, and each of these domains is encoded by a separate exon. The multi-modular structural organization of CCN1 protein suggests that its diverse biological functions are programmed by the combinatorial actions of individual domains, either acting independently or interdependently [49,50]. CCN1 exerts a range of functions by interacting with numerous integrins, in a cell type and function-specific manner [42].

In human skin, CCN1 is predominantly expressed in dermal fibroblasts [51,52]. Interestingly, CCN1 is markedly induced by ROS [35] and significantly elevated in dermal fibroblasts in aged human skin *in vivo* [51,53,54]. In cultured human dermal fibroblasts, elevated expression of CCN1 not only inhibits the expression of type I procollagen, the major structural protein in skin, but also concurrently increases the expression of multiple matrix metalloproteinases (MMPs) and cytokines [35,51,53-55]. Mechanistically, elevated CCN1 impairs TGF-β signaling by down-regulation of TGF-β type II receptor (TβRII) expression, thereby contributes to reduced type I procollagen expression. Additionally, elevated CCN1 induces transcription factor AP-1, which functions to stimulate the expression of multiple MMPs and cytokines.

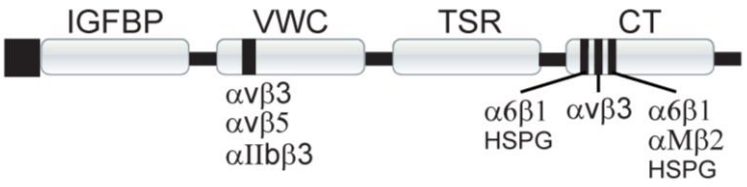

Figure 1. CCN1 structural and functional domains. Each domain is depicted as a grey box with the domain name shown. The black box represents the secretory signal peptide. Integrin/HSPGs binding sites are marked as stripes.

Emerging evidence indicates that CCN1 functions as a novel mediator of collagen homeostasis [17,53,56,57]. Elevated CCN1 in human dermis alters expression of numerous secreted proteins, which together have deleterious effects on dermal microenvironment, and thus impair the structural integrity and function of skin. CCN1-induced alteration of dermal microenvironment referred as "Age-Associated Secretory Phenotype (AASP)" [17,53]. AASP includes: 1) reduced expression skin ECM components, such as collagens related to dermal thinning; 2) increased expression of collagen-degrading enzymes, such as multiple MMPs related to fragmentation of dermal ECM; and 3) increased expression of pro-inflammatory cytokines, such as multiple interleukins related to oxidative stress tissue microenvironment. Importantly, CCN1-induced AASP is observed in aged human dermis *in vivo*, and logically could account for many of the characteristic features of human skin. Therefore, progressive development of CCN1-induced AASP may drive loss of tissue structure and function, which are characteristic features of aging in all tissues including skin.

CCN1 is also significantly induced by acute UV irradiation in human skin *in vivo*, and in UV-irradiated human dermal fibroblasts *in vitro* [54,58]. Knockdown of CCN1 by CCN1 siRNA significantly prevents UV irradiation-induced inhibition of type I procollagen and up-regulation of MMP-1, indicating CCN1 functions as a key mediator of UV-induced aberrant collagen homeostasis. The primary mechanism of CCN1 induction by UV irradiation indicated that CCN1 is largely regulated at transcriptional level. CCN1 proximal promoter contains a consensus binding site for transcription factor AP-1, which is required for promoter activity. UV irradiation increases the binding of AP-1 transcription factors, c-Jun/c-Fos, to CCN1 AP-1 binding site. Functional blockade of c-Jun or knockdown of c-Jun significantly reduces the UV irradiation-induced activation of CCN1 promoter and CCN1 gene expression. These data show that CCN1 is transcriptionally regulated by UV irradiation through transcription factor AP-1, and mediates altered collagen homeostasis that occurs in response to UV irradiation in human dermal fibroblasts.

CCN1 contributes to human skin connective tissue aging, at least in part, through up-regulation of multiple proinflammatory cytokines such as interleukin 1β (IL-1β) [53,57,59]. Both CCN1 and IL-1β are strikingly induced by ROS and acute UV irradiation in the dermis of human skin *in vivo* and *in vitro*. CCN1 markedly induces IL-1β, which in turn mediates CCN1-induced aberrant collagen homeostasis. Furthermore, knockdown of CCN1 significantly reduces UV/ROS-induced IL-1β, and therefore partially prevents UV/ROS-induced aberrant collagen homeostasis. These data suggest that induction of CCN1 by UV/ROS is responsible for up-regulation of IL-1β, which mediates UV/ROS-induced aberrant collagen homeostasis. Elevated expression of CCN1 by UV/ROS is likely an important mediator of inflammation and connective tissue damage in aged human skin.

CCN1 is markedly induced by ROS and mediates aberrant collagen homeostasis in human dermal fibroblasts. It has been reported that antioxidant, N-acetyl-L-cysteine, significantly reduced CCN1 expression and prevented ROS-induced loss of type I collagen in both human skin *in vivo* and human dermal fibroblasts *in vitro* [35]. ROS increased c-Jun, a critical member of transcription factor AP-1 complex, and increased c-Jun binding to the AP-1 site of the CCN1 promoter. Functional blocking of c-Jun significantly reduced CCN1 promoter and gene expression and thus prevented ROS-induced aberrant collagen homeostasis. These data suggest that targeting the c-Jun/CCN1 axis may provide clinical benefit for ROS-mediated connective tissue aging in human skin.

As replicative senescence is a model of cellular aging, CCN1 mRNA and protein levels are markedly elevated in replicative senescent dermal fibroblasts [56]. Replicative senescent dermal fibroblasts also express significantly reduced levels of type I procollagen and increased levels of MMP-1, as observed in aged human skin *in vivo*. Knockdown of elevated CCN1 by CCN1 siRNA in senescent dermal fibroblasts partially normalizes both reduced type I procollagen and elevated MMP-1 expression. These data further support an important role of CCN1 in regulation of collagen homeostasis, and elevated CCN1 functions as an important mediator of collagen loss, which is observed in aged human skin.

It has been shown that CCN1 exerts a range of functions primarily through interactions with integrins in a cell-type, function-specific manner [36,42,60]. CCN1 has been reported to interact with integrins to increase intracellular levels of ROS, which function as important effectors of CCN1 functions [43,61]. In human skin dermal fibroblasts, secreted CCN1 physically interacts with αVβ3 integrin to activate focal adhesion kinase (FAK) and paxillin, two key effectors of integrin signaling [55]. These data suggest that the ability of CCN1 to induce AASP 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-κb signaling, which up-regulate MMPs and proinflammatory cytokines, including IL-1β and IL-6 [39,61]. Therefore, it is conceivable that elevated CCN1 up-regulates MMPs and cytokines through activation of integrin and/or ROS-mediated activation of MAKP and NF-κb signaling in aged human skin dermal fibroblasts. It is also noteworthy that elevated IL-1β and IL-6 drives not only up-regulation of MMPs but also down-regulation of collagen synthesis, since these cytokines have been shown to negatively regulate collagen expression [62-64]. Interestingly, CCN1 is not only increases intracellular levels of reactive oxygen species, [61], but also induced by oxidative stress [35], suggesting that a positive feedback loop may cause sustained elevation of ROS and CCN1 in fibroblasts in aged human skin.

The precise molecular mechanisms by which CCN1 regulates diverse AASP-related genes remain to be determined. CCN1 interacts with distinct integrins in a cell type-, function-specific manner to mediate disparate biological activities. In skin fibroblasts, CCN1 is able to interact αVβ3 integrin to upregulate MMPs [55] and inflammatory cytokines [59] through activation of transcription factor AP-1 [17,51]. It is well-documented that integrin mediated pathway ultimately lead to the stimulation of AP-1 [65-67]. AP-1 not only functions as a strong inducer of multiple MMPs [68-70], inflammatory cytokines [71], and also negatively regulates type I procollagen expression by transrepression of Smad3 [72-74]. Therefore, it is concealable that the ability of CCN1 regulates AASP-related genes is likely mediated by integrin αVβ3-medaied activation of AP-1 pathway. Obviously, it will be of great interest to investigate how elevated CCN1 regulates diverse AASP-related genes and contributes to human skin aging.

4. Conclusion and outlook

Figure 2 depicts a model in which elevated CCN1 in aged dermal fibroblasts contributes to human skin connective tissue aging through secreting age-associated secretory phenotype (AASP) and creating age-associated dermal microenvironment (AADM). Unlike other organs, human skin is exposed to reactive oxygen species (ROS) generated from both environmental sources such as solar ultraviolet irradiation and endogenous oxidative metabolism. Chronic exposure to ROS up-regulates CCN1 expression. Through interaction with integrins elevated CCN1 impairs dermal fibroblast production of collagen by inhibiting TGF-β signaling, and promoting production of MMPs as well as proinflammatory cytokines. These alterations lead to thin and fragmented dermal collagenous ECM, the characteristic features of aged human skin. Elevated expression of CCN1 in human dermal fibroblasts acts through multiple pathways to promote AADM and thus contribute to skin connective tissue aging: 1) impairment of TGF-β signaling by down-regulation of TβRII, and thus contributes to age-associated thinning of the dermis [51]; 2) induction of multiple MMPs via up-regulation of transcription factor AP-1, a major regulator of multiple MMPs, and thus contributes to age-associated ECM fragmentation [51,53,75]; and 3) elevation of multiple pro-inflammatory cytokines, and thus contributes to age-associated inflammatory microenvironment (inflammaging) [53,76]. In addition, CCN1 is not only increases intracellular levels of ROS [61], but also induced by oxidative stress [35], and thus forming a positive feedback loop between ROS and CCN1. The self-perpetuating positive feedback loop causes sustained elevation of ROS and CCN1 in fibroblasts in aged human skin.

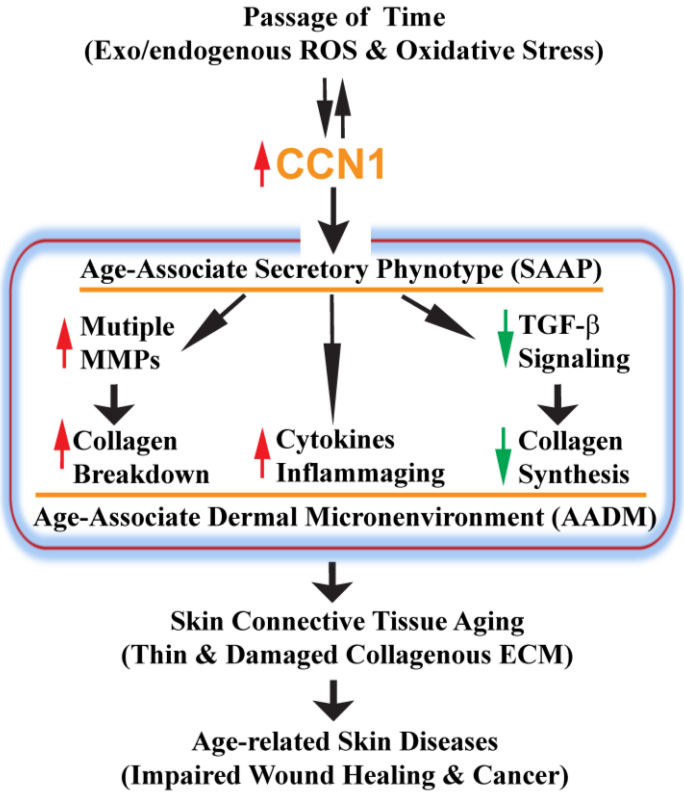

Figure 2. Proposed model in which elevated CCN1 in aged dermal fibroblasts contributes to human skin connective tissue aging through secreting age-associated secretory phenotype (AASP) and creating age-associated dermal microenvironment (AADM).

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Conflict of interest

The authors declare that they have no competing interests.

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