Cysteine-rich protein 61 (CCN1) and connective tissue growth factor (CCN2) at the crosshairs of ocular neovascular and fibrovascular disease therapy

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Abstract

The vasculature forms a highly branched network investing every organ of vertebrate organisms. The retinal circulation, in particular, is supported by a central retinal artery branching into superficial arteries, which dive into the retina to form a dense network of capillaries in the deeper retinal layers. The function of the retina is highly dependent on the integrity and proper functioning of its vascular network and numerous ocular diseases including diabetic retinopathy, age-related macular degeneration and retinopathy of prematurity are caused by vascular abnormalities culminating in total and sometimes irreversible loss of vision. CCN1 and CCN2 are inducible extracellular matrix (ECM) proteins which play a major role in normal and aberrant formation of blood vessels as their expression is associated with developmental and pathological angiogenesis. Both CCN1 and CCN2 achieve disparate cell-type and context-dependent activities through modulation of the angiogenic and synthetic phenotype of vascular and mesenchymal cells respectively. At the molecular level, CCN1 and CCN2 may control capillary growth and vascular cell differentiation by altering the composition or function of the constitutive ECM proteins, potentiating or interfering with the activity of various ligands and/or their receptors, physically interfering with the ECM-cell surface interconnections, and/or reprogramming gene expression driving cells toward new phenotypes. As such, these proteins emerged as important prognostic markers and potential therapeutic targets in neovascular and fibrovascular diseases of the eye. The purpose of this review is to highlight our current knowledge and understanding of the most recent data linking CCN1 and CCN2 signaling to ocular neovascularization bolstering the

potential value of targeting these proteins in a therapeutic context.

Keywords CCN1 · CCN2 · Extracellular matrix · Neovascularization · Retinopathy · Ischemia

Abbreviations

AGE	Advanced glycation end-product
AMD	Age-related macular degeneration
Ang	Angiopoietin
CNV	Choroidal neovascularization
ECM	Extracellular matrix
GCL	Ganglion cell layer
GFP	Green fluorescent protein
IGF	Insulin-like growth factor
IPL	Inner plexiform layer
INL	Inner nuclear layer
MMP	Matrix metalloproteinase
OIR	Oxygen-induced retinopathy
PDR	Proliferative diabetic retinopathy
RPE	Retinal pigment epithelium
ROP	Retinopathy of Prematurity
ROS	Reactive oxygen species
TGF	Transforming growth factor
TNF	Tumor necrosis growth factor
VEGF	Vascular endothelial growth factor

Introduction

Extracellular matrix (ECM) proteins are structural and informational entities supporting key signaling events involved in the regulation of endothelial cell differentiation and function during developmental morphogenesis, in response to injury and in pathological conditions. Constitutively expressed ECM proteins such as the collagens, proteoglycans, elastin

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and glycoproteins provide the mechanical scaffolding within which tissues and organs are built. Cells surface receptors typified by integrins and non-integrin receptors anchor cells in such ECM and provide cues for tissue morphogenesis and maintenance of a differentiated state and enhance tissue repair after injury (Bou-Gharios et al. 2004). During angiogenesis, stimulated endothelial and mural cells directly modulate the physico-mechanical properties of the surrounding matrix determined by its composition, to support new vessel formation and maturation through changes in their synthetic and differentiation phenotype (Vogel 2006). The active participation of these molecules is inescapably evident in blood vessel formation and regeneration under normal and pathological conditions.

In recent years, research interest in the regulation and function of ECM proteins has increasingly been focused on a subset of ECM proteins that appear only transiently in the extracellular environment during specific developmental or pathological events. These molecules named matricellular proteins, do not subserve a structural/physical role in the extracellular environment but mainly function as upstream regulators of synthesis and degradation of the constitutively expressed ECM proteins and influence cell fate and function (Bornstein and Sage 2002). Among known matricellular proteins are the prominent and functionally vital members of the CCN protein family, cysteine-rich protein 61 (Cyr61) now known as CCN1 and connective tissue growth factor (CTGF) also known as CCN2 (Perbal 2013).

CCN1 and CCN2 are immediate early gene-encoded non-structural bioactive ECM molecules bridging the functional divide between structural macromolecules and growth factors, cytokines, proteases, and other related proteins (Brigstock 2003; Leask and Abraham 2006). CCN1 and CCN2 exhibit ECM-like structural features and growth factor-like activities including modulation of cell motility, adhesion, proliferation, predisposition to apoptosis and reprogramming of gene expression (Hall-Glenn et al. 2012; Jun and Lau 2011). As such, these molecules are prime candidates for the modulation of blood vessel formation and regeneration during development and disease. The CCN1 and CCN2 proteins exhibit both overlapping and distinct tissue distributions and functions but, structurally, they share a high content and an absolute conservation of the positions of 38 cysteine residues in their primary sequences as well as conservation of structural motifs and domains derived from known eukaryotic modules (Holbourn et al. 2009). CCN1 and CCN2 regulate, although sometimes differentially, a variety of processes including angiogenesis, fibrosis, and chondrogenesis either by directly modulating cell behavior and function or indirectly by altering the expression and/or function of selective genes including vascular endothelial growth factor (VEGF)-A and VEGF-C, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase (TIMP)-1, urokinase plasminogen

activator (uPA), plasminogen activator (PAI)-1, $\alpha 1$ and $\alpha 2$ chains of type I collagen and integrins α_3 and α_5 .

CCN1 and CCN2 have not only overlapping functions but also conflicting biological activities. The most well-known functional conflict is their role in the angio-fibrotic switch (Kuiper et al. 2008a) i.e., an overbalance of CCN2 in the eye halts angiogenesis presumably promoted by CCN1 and VEGF, and begins the process of fibrosis. Other opposing biological activities involve the ability of CCN2 to initiate and propagate fibrotic reactions while CCN1 rather promotes resolution of fibrosis (Kim et al. 2013). Similarly, while CCN2 is required as a basic need for cells such as fibroblasts to survive and properly participate in embryologic functions (Kennedy et al. 2007), CCN1 seems to reduce survival of the otherwise apoptosis-resistant skin fibroblasts (Juric et al. 2012). The biological properties of CCN1 and CCN2, whether they overlap or conflict, will be further expanded upon below in the context of neovascular and fibrovascular diseases of the eye.

An interesting example of the effects of CCN1 and CCN2 dysregulation is the phenotype of knockout mouse lines bearing null mutations in either CCN1 or CCN2. As suggested by the phenotype of CCN1-deficient mice, CCN1 is critical for angiogenesis and proper blood vessel formation during development (Mo et al. 2002). Severe atrioventricular septal defects were observed in CCN1 null mice caused by an impaired ECM remodeling during development (Mo and Lau 2006). Interestingly, healthy adult mice do not typically express CCN1 in the cardio-vascular system unless challenged with a disease state suggesting that CCN1 plays mainly developmental and repair roles (Lee et al. 2007; Schober et al. 2002).

Likewise, CCN2 knockout mice were nonviable, in this case due to respiratory failure caused by severe skeletal deficiencies although concomitant vascular defects were not completely ruled out (Ivkovic et al. 2003). Indeed, CCN2 deletion led to reduced vascularization during endochondral ossification, indicating that CCN2 plays a role in maintaining the normal ECM composition necessary for angiogenesis. As extracellular proteins that interact with and regulate a number of other extracellular proteins, CCN1 and CCN2 are necessary for the creation of a balanced ECM environment, the disruption of which can affect any range of processes that rely on the ECM for support and development. Here, we use the implications of CCN1 and CCN2 knockout mice as a springboard to examine the role of these molecules in retinal vascular development and diseases.

Requirement of CCN1 and CCN2 in developmental retinal angiogenesis

The retinal vascular supply is derived from two separate vascular systems: (i) the choroids, which provide oxygen to the outer retina, is highly vascularized but it has a small

arteriovenous oxygen difference (Provis 2001), and (ii), the inner retinal vasculature which is comparatively sparse to allow unperturbed passage of light to photoreceptors but it has a significant arteriovenous oxygen difference (Fig. 1). Unlike the choroidal circulation, which is controlled by sympathetic innervation and irrigates the outer part of the retina, the retinal circulation has no autonomic innervation and is controlled essentially by local factors (Saint-Geniez and D'Amore 2004). Similarly, the retinal vasculature has blood–brain barrier-like properties while choroidal vessels are of the fenestrated type.

The mammalian eye derives embryologically from three embryonic tissue sources, the neural ectoderm, the surface ectoderm, and the periocular mesenchyme (Heavner and Pevny 2012). Interestingly, during development, the inner retinal vasculature is absent, and oxygenation of the retina is provided uniquely by choroidal and hyaloid vessels (Zhu et al. 2000). Hyaloid vessels form a complex of intraocular vessels that penetrate the retina at the optic disc and branch anteriorly through the vitreous to the lens. Hyaloid vessels then regress progressively by apoptosis as the retinal vasculature develops by angiogenesis in a synchronized manner suggesting that overlapping signals control both processes simultaneously. Failure of the hyaloid vessels to regress is a characteristic feature of Norrie disease, an X-linked congenital syndrome due to mutations in the norrie disease protein (NDP), a novel ligand for the frizzled-4 receptor that activates the canonical Wnt pathway (Hendrickx and Leyns 2008). Concordantly, mutations in frizzled 4 cause familial exudative vitreoretinopathy, a developmental disorder associated with persistence of hyaloid vessels and aberrant retinal vascularization (Warden et al. 2007). Disease symptoms

include severe intraocular hemorrhage, retinal detachment and eventually blindness.

The onset of retinal vasculature development and hyaloid vessel regression occurs in humans around mid-gestation and in mice around birth. A superficial vascular plexus (containing both arteries and veins) emerges from the optic nerve head and propagates in the ganglion cell layer (GCL) across the inner surface of the retina with the more mature vessels at the central region and less mature vascular tubes toward the periphery. Once the primary plexus is completely established, branches emerge from it and dive vertically into the deeper retina to form two parallel interconnected intermediary and deeper capillary plexuses. The deeper networks of the retinal vasculature are believed to form by sprouting angiogenesis while, as suggested by some studies (Lutty et al. 2010), the primary superficial vascular plexus is formed by vasculogenesis and/or haemavascuogenesis although there has been no reported evidence of free-standing progenitors distal to the advancing primary vascular network in the developing mouse retina.

During development, endothelial cells respond to a variety of growth factors and/or cytokines that promote their migration, guidance, growth and ultimately vessel maturation which involves pruning of the primitive vascular network (Ishida et al. 2003), production and assembly of a basement membrane, and recruitment of accessory mural cells (Das and McGuire 2003). Specific VEGF isoforms (e.g., VEGF¹⁶⁴) as well as insulin-like growth factor (IGF)-I are commonly viewed as central coordinators of retinal vascular development (Stalmans 2005). Initially, retinal astrocytes, localized in the ganglion cell layer, act as the primary proangiogenic cell type required to establish the retinal vascular network. They invade the retina from the optic nerve head as a proliferating population of cells and

Fig. 1 Schematic representation of the different cellular and acellular layers of the retina and their vascularization pattern. The retinal vasculature composed of the superficial, intermediate and deep capillary plexuses

has a blood–brain barrier-like properties, no autonomic innervation and is controlled by local growth factors. The choriocapillaris irrigates the outer part of the retina and is under a sympathetic innervation

spread across the inner surface of the retina, creating a template for the developing retinal vasculature which follows in their wake. As they migrate into the hypoxic environment of the avascular retina, astrocytes produce and create a VEGF gradient which at least partly provides a directional stimulus for retinal vascularization (Nakamura-Ishizu et al. 2012).

The importance of CCN1 and CCN2 proteins in the retina is underscored by their dynamic expression and localization during vascular development. During mouse development, CCN1 gene expression peaks as the chorioallantoic plate is invaded by fetal blood vessels from the allantois with continuing expression throughout the developmental stages of the cardiovascular system (Hasan et al. 2011). As CCN1-deficient mice die prematurely before development of mouse retinal vessels which occurs postnatally in mice, the vascular phenotype in the retina of these animals could not be assessed. Using transgenic mice expressing the green fluorescent protein (GFP) gene under the control of the CCN1 promoter, we found that CCN1 was dynamically expressed postnatally in the mouse eye and that its expression was transient and largely confined to ocular vasculature. CCN1 expression is associated with hyaloid vessels and shifts to the retinal vasculature as it emerges from the optic disc (Fig. 2). CCN1 levels peak between P2 and P4, when the budding of superficial vessel begins and between P8 and P12 when the secondary deep

layer of the retinal vasculature starts extending and growing radially outward toward the inner nuclear and outer plexiform layer (Hasan et al. 2011). CCN1 expression then progressively declines and becomes barely detectable once the adult vasculature is completely established. Vascular cells including endothelial cells, pericytes and possibly retinal pigment epithelial (RPE) cells and active astrocytes are sources of CCN1 (Table 1).

Interestingly, CCN2 expression pattern is somewhat similar to that of CCN1. CCN2 expression coincides with the formation of primary, intermediary and deep capillary plexuses (Chintala et al. 2012). When the retinal vasculature is completely established, the expression of CCN2 decline but maintains relatively high basal levels in the adult vasculature. CCN2 expression has been localized in cells of the growing superficial vascular plexus around the optic nerve head but also in the intermediate and deep plexuses within the inner nuclear layer. Angiogenic cells and especially Muller cells, RPE cells and the tip cells projecting filopodia at vascular fronts, highly expressed CCN2 while astrocytes did not appear to be a source of CCN2 (Kita et al. 2007; Watanabe et al. 2005). Interference with CCN2 expression did not inhibit but only delayed sprouting and complete formation of the superficial capillary plexus suggesting that adequate levels of CCN2 are needed for normal retinal vessel growth and patterning (Chintala et al. 2012).

Fig. 2 Expression pattern of CCN1 in the retina of a transgenic mouse expressing green fluorescent protein (GFP) under the control of a 5.4-kb fragment of the CCN1 promoter. GFP expression recapitulates CCN1 expression pattern during postnatal development of the retinal vasculature. **a** immunostaining of flat mounted retinas at postnatal day (P) 1 with antibodies against either alpha1 chain of type 4 collagen (Col4A1) or GFP antibodies. GFP localizes essentially in the hyaloid vessels. **b** immunostaining of flat mounted retinas at P7 with antibodies against either Col4A1 or GFP. Note that CCN1 appears in developing retinal vessels replacing the hyaloid vessels that has regressed. **d** immunostaining of a cross section of the retina at P12 showing localization of GFP in the superficial, intermediate, deep capillary plexuses. Note the GFP staining is confined to the retinal and choroidal vasculatures only

Table 1 Cellular sources of CCN1 and CCN2 in the retina

	CCN1	CCN2
Ganglion cells	–	–
Astrocytes	+	–
Pericytes	++	++
Endothelial cells	++++	++++
Smooth muscle cells	++	++
RPE	++	++
Horizontal cells	–	–
Müller cells	–	+
Neurons	–	–
Neuronal progenitors	+	?

CCN1 and CCN2 in retinopathy of prematurity

Retinopathy of Prematurity (ROP) is a multistage disease that affects at-risk preterm infants. Risks for adverse outcomes increase with decreasing gestational age. A disrupted oxygen environment in the retina is a key factor in the onset of a vaso-obliteration of the retina which creates an ischemic environment conducive to blood vessel regrowth. However, the subsequent neovascular response generates disorganized, leaky and tortuous vessels prone to exudation and fibrosis, ultimately breaking through the inner limiting membrane into the vitreous and causing hemorrhage, tractional retinal detachment and vision loss (Hartnett and Penn 2012). Severe cases of ROP are caused by an imbalance in pro-and anti-angiogenic factors and the failure of neovascularized regions to regress (Martinez-Castellanos et al. 2013). Hypoxia plays a key role in this dysregulation as it promotes hypoxia-induced transcription factors and reactive oxygen species (ROS), leading to abnormal and aggressive growth of blood vessels (Li et al. 2012). Current treatment focuses on laser surgery and pre-surgery application of bevacizumab, an anti-VEGF antibody, in an attempt to control and stem neovascularization (Mintz-Hittner 2012).

Expression of CCN1 in preterm infants with ROP is unknown, but recent research using a mouse model subjected to oxygen-induced retinopathy (OIR) suggests that the expression of the CCN1 gene is altered during vaso-obliteration and vaso-proliferation phases of ROP (Hasan et al. 2011). Overall, the expression of CCN1 is commonly repressed during tissue involution, in avascular tissues and under conditions associated with vaso-obliteration (Perkowski et al. 2003; Hilfiker-Kleiner et al. 2004; Jin et al. 2005). In the retina, hyperoxia induces vaso-obliteration and cessation of vascular development in the capillary beds (Hasan et al. 2011). This occurs concomitantly with a significant decrease of the levels of angiogenic and permissive factors such as VEGF and IGF-1 which participate in the establishment and stabilization of retinal vascular networks, and those of TNF- α , which influence vascular growth through regulation of endothelial cell

cycle (Campochiaro 2000). Remarkably, restoration/ectopic expression of the CCN1 protein levels in OIR mice reduced vaso-obliteration following hyperoxic insult and promoted normalization of the retinal vasculature during the ischemic phase (Hasan et al. 2011). Jin et al. demonstrated that recombinant CCN1 promoted cell survival and increased resistance to hyperoxia-induced cell death which is consistent with a cytoprotective effect of CCN1 (Jin et al. 2005).

Paradoxically, CCN2 effects on retinal neovascularization appeared to be antithetic to those of CCN1. In mice subjected to OIR, the CCN2 gene was overexpressed in neovascular areas and CCN2 protein localized within neovascular tufts (Chintala et al. 2012). Unsurprisingly, silencing of CCN2 reduced neovascularization in the mouse OIR model. However, a study by Kuiper et al. showed that neovascularization was not altered in CCN2 hemizygote mice subjected to either OIR or laser-induced choroidal neovascularization as compared to wild type mice (Kuiper et al. 2008b). Although the latter observations may appear to challenge the concept of a role of CCN2 in pathological angiogenesis, it actually underscores the importance of CCN2 levels and/or localized presence in promoting neovascular growth. A potential overlap and/or functional compensation between CCN1 and CCN2 cannot be ruled out either. The observations that inhibition of CCN2 expression delayed but did not completely inhibit retinal vessel formation during development and that CCN2 is highly pro-angiogenic when administered exogenously in the cornea or other tissues are in support of a significant role of CCN2 in neoangiogenesis (Babic et al. 1999; Kuiper et al. 2008a; Shimo et al. 1999).

CCN1 and CCN2 in proliferative diabetic retinopathy

Similar to ROP, proliferative diabetic retinopathy (PDR) culminates in retinal scarring and detachment caused by recurring neovascularization (Aiello et al. 1998). The additional contributor to the pathology of PDR is hyperglycemia-induced change in cellular and ECM protein composition in the eye and the subsequent cellular insult by advanced glycation end (AGE) products. In the early non-proliferative stages of diabetic retinopathy, hyperglycemia and AGE products trigger vascular cell apoptosis and pericyte death. Oxidative stress and inflammation further attenuate vascular wall integrity and increase vascular permeability, occlusion, and retinal neovascularization (Geraldes et al. 2009; Dulmovits and Herman 2012). The upregulation of numerous factors, both angiogenic and inflammatory, has been implicated in the pathogenesis of PDR. VEGF, the most active promoter of aberrant vasculature and inflammatory factors including the interleukins, TNF- α , IGF and angiopoietins (Ang-2), among many others have all been implicated in the pathogenesis of clinical PDR (Das and McGuire 2003; Yang et al. 2007).

While the specific role of CCN1 in PDR is still under speculation, it has been confirmed that CCN1 is a gene target and downstream effector of AGEs and hyperglycemia (Hughes et al. 2007; Liu et al. 2008). Yet even this fact raises many more questions than it answers, as CCN1 has been known to be spliced in alternate forms and/or degraded posttranslationally under conditions of inflammation or hypoxia (Hirschfeld et al. 2009). Not surprisingly, it is alternate truncated forms of CCN1 protein that have been found to be upregulated in PDR (Choi and Chaqour, Personal communication). Truncated forms of CCN1 exhibit biological activities different from the parent CCN1 protein and become surrogate markers of the CCN1 activity under diseased conditions. Thus, there seems to be a correlation not between CCN1 and PDR, but between the pathological alterations of the CCN1 protein and PDR.

Because CCN2 plays a major role in maintaining the ECM and wound healing, greater emphasis has been placed on its potential involvement in ECM remodeling associated with PDR (Twigg et al. 2001). In diabetic CCN2 hemizygous mice, basal lamina thickening of retinal capillaries was found to be reduced compared to diabetic wild type mice (Kuiper et al. 2008b). Similarly, a study by Van Geest showed that CCN2 levels correlated positively, and VEGF levels correlated negatively with the degree of fibrosis in patients with PDR (Van Geest et al. 2013). Genetic modification of CCN2 gene expression in mice provided controversial information on the role of CCN2 in tissue fibrosis. The traditional knockout of CCN2 resulted in perinatal lethality due to skeletal deformity, a phenotype similarly observed in knockout mice for constitutively expressed ECM proteins such as the collagens and proteoglycans, suggesting a possible regulatory relationship between CCN2 and other ECM proteins (Arnott et al. 2011; Ivkovic et al. 2003). However, transgenic models of CCN2 overproduction in various tissues exhibited various phenotypes including no fibrotic reaction, mild fibrosis and clear fibrotic phenotypes depending on the CCN2 levels (Doherty et al. 2010). Interestingly, *in vitro* assays showed that CCN2 may physically interact with VEGF and that the CCN2-VEGF complex has a reduced proangiogenic activity (Inoki et al. 2002). Subsequently, the concept of a fibro-angiogenic switch has been put forward. Such a switch may occur as an excess of CCN2 suppresses angiogenesis through formation of inactive CCN2-VEGF complexes and activates fibrosis instead (Kuiper et al. 2008a). This concept was very appealing as it provided a reasonable explanation for the occurrence of the fibrotic phase in combination with fibrovascular contraction causing hemorrhages, retinal detachment and vision loss during advanced PDR. Complicating the matter was a finding by Hinton et al. that only an NH2 terminal fragment of the CCN2 molecule was stably expressed under PDR conditions (Hinton et al. 2004). However, the *in vivo* biological activities of such a truncated variant and the extent of its interactivity with and inhibition of VEGF are not known and should be

investigated. In any case, it is clear that the *in vivo* activities of CCN2 during PDR are context-dependent and that CCN2 unequivocally affects the development and progression of the disease.

CCN1 and CCN2 in age-related macular degeneration and choroidal neovascularization

Neovascular or exudative age-related macular degeneration (AMD) is the leading cause of vision loss in the elderly. AMD occurs as a result of abnormal blood vessel growth in the choriocapillaris (i.e., choroidal neovascularization or CNV), ultimately leading to protein leakage below the macula, a region near the center of the retina with specialized cells for high-acuity vision. The macula receives oxygen and nutrients through the choriocapillaries, which is separated from the macula by Bruch's membrane and the retinal pigment epithelial layer (Caprara and Grimm 2012). CNV is also associated with pathological myopia, a leading cause of vision impairment in patients younger than 50 years (Neelam et al. 2012). Although the cellular and molecular bases for CNV are not entirely understood, a disruption of Bruch's membrane commonly caused by a traumatic break, degeneration of the retinal pigment epithelium, tissue traction, and/or inflammation can lead to development of CNV. Subsequently, choriocapillary endothelial cells, pericytes, fibroblasts, and inflammatory cells invade the subretinal space causing abnormal choroidal neovessel growth and tissue remodeling/scarring that culminates into the formation of a choroidal neovascular membrane. At the molecular levels, oxidative stress and other age-related changes can affect the metabolism of the retinal pigment epithelial layer, causing accumulation of proteins, lipids, and AGEs above Bruch's membrane. Over time, protein deposits known as drusen may accumulate over Bruch's membrane, further adding to the dysregulated environment already created by decreasing RPE layer metabolism. As drusen accumulates, Bruch's membrane thickens and becomes less permeable, slowing the transport of metabolites between the retina and the choroid (Barzegar-Befroei et al. 2012). The risk of neovascularization increases as the extracellular environment in Bruch's membrane, the macula, the RPE layer, and the choriocapillaries becomes disorganized. RPE cells have the ability to proliferate, migrate and differentiate into myofibroblasts or mesenchymal-like cells forming epiretinal membranes within a provisional ECM. These membranes exert upward tensional forces on the attached underlying retina, leading to retinal detachment and ultimately blindness.

What particular role CCN1 and CCN2 play in this process remains to be uncovered. A study by He et al. showed an intensive immunoreactivity to CCN2 antibodies in surgically excised human choroidal neovascular membranes especially within choroidal endothelial cells and RPE cells (He et al. 2003). CCN2 protein was detected in neither the RPE nor

choroidal endothelial cells in adult normal retina suggesting that CCN2 has the ability to activate the underlying choroidal endothelium. Our studies in the mouse CNV model which mimics the human condition albeit imperfectly, through injury to the outer retina and break in Bruch's membrane, showed that both CCN1 and CCN2 were upregulated at the site of injury (Caballero et al. 2011). In particular, CCN2 promoted changes of RPE behavior and function including their differentiation state, proliferation, migration, matrix synthesis, enzyme production, and contraction. A major pathogenic role of these molecules was then postulated with CCN1 as a potential mediator of choroidal endothelial cell growth dysregulation and CCN2 as mediator of ECM remodeling resulting in the development of fibrovascular subretinal membranes. Interestingly, non-selective inhibition of CCN1 and CCN2 gene expression through cytoskeletal cell disruption reduced CNV lesion size and fibrosis by up to 60 % in the mouse model of CNV (Caballero et al. 2011). Clearly, CCN1 and CCN2 hold a particular position at the crossroad of neoangiogenesis and fibrosis associated with CNV.

Glaucoma

The most important risk factor for glaucoma, the leading cause of blindness in the world, is an increase in the pressure of aqueous humor through the blockage of aqueous drainage pathways, which include the trabecular meshwork and uveoscleral outflow (Quigley 2011; Tamm 2009). The onset of the disease is characterized by the gradual build up of intraocular pressure, either through an overproduction of aqueous humor, resistance in draining aqueous humor, or both, which leads to painless and gradual damage to the optic nerve (Kwon et al. 2009). Our laboratory has previously reported that both the CCN1 and CCN2 genes are sensitive to mechanical forces and to changes in the mechanical properties of the cell environment (Han et al. 2003; Hanna et al. 2009). Thus, intraocular pressure changes are conducive to aberrant increases of CCN1 and CCN2 levels and subsequent dysregulation of ECM protein expression and turnover.

The most commonly prescribed treatment in neovascular glaucoma cases is based on the use of prostaglandins, which mediate structural changes in the ECM of the ciliary muscle, and reduce intraocular pressure by increasing uveoscleral outflow (Dams et al. 2013). If administered successfully, prostaglandins such as Bimatoprost, Latanoprost, and Travoprost can achieve a 30 % reduction of intraocular pressure compared to pre-treatment levels (Schwartz and Budenz 2004). Interestingly, three common prostaglandins, prostaglandin F₂ α , Butaprost, and Bimatoprost, have been shown to upregulate the CCN1 and CCN2 gene expression through various pathways (Liang et al. 2003). Prostaglandin F₂ α upregulation of CCN1 is dependent on the Rho pathway while prostaglandin EP2 signals through

prostanoid receptors. It was suggested that CCN1 promotes structural changes that increase uveoscleral outflow. On the other hand, CCN2 and its fibrogenic partner TGF- β have been found to be upregulated in glaucoma, and play a role in abnormal ECM accumulation (Fuchshofer et al. 2009). CCN2 was found to be constitutively expressed in the trabecular meshwork and plays a critical role in the physiological regulation of aqueous humor outflow (Chudgar et al. 2006).

Molecular activities of CCN1 and CCN2 in neovascular and fibrovascular diseases

The functions of CCN1 and CCN2 are achieved in part by direct interaction with a variety of cell-surface receptors, with the resulting engagement of specific signal transduction pathways, activation of transcription factors, transactivation of a set of effector genes and subsequent change of cellular response/behavior. The signaling cascades activated by CCN1 and CCN2 at the cell membrane and the subsequent cellular responses have been extensively and elegantly described in other review papers and will not be described here (Chen and Lau 2009; Chen and Du 2007; Mason 2009). Candidate signaling mechanisms utilized by CCN1 and CCN2 include the activation of ion channels, mitogen activated protein kinases and small GTPases. However, it is noteworthy that separate linear pathways linking events at the cell membrane to changes in gene expression and cell function and behavior may be an oversimplification. Instead, complex and interdependent signaling networks co-exist simultaneously.

CCN1 and CCN2 exhibit a high affinity for multiple integrins found in endothelial and mural cells (Chen and Lau 2009; Chen et al. 2004). This is consistent with the similarity of phenotypes between mice deficient in CCN1 and certain integrin subunits, (e.g., $\alpha_v\beta_3$ and $\alpha_5\beta_1$). Mice lacking the α_v chain for instance are embryonic lethal due to widespread vascular malformations and hemorrhage (Bader et al. 1998). The $\alpha_v\beta_3$ integrin is highly expressed in human endothelial cells and has been shown to mediate human endothelial cell migration in vitro and in vivo. During pathologic vascularization $\alpha_v\beta_3$ integrin plays a central role in the aberrant growth of new vessels by virtue of its ability to bind VEGF and even to associate with the VEGF receptor to promote enhanced responses to the VEGF ligand (De et al. 2005). Of particular interest is that CCN1 is also able to block cell interaction with $\alpha_v\beta_3$ integrin in a context environment enriched with vitronectin C (Francischetti et al. 2010). As such, CCN1 may interfere with VEGF signaling reducing its permissive and pro-growth effects and providing an environment conducive for normal growth of blood vessels under pathological conditions (Hasan et al. 2011). Likewise, CCN2 may play a significant role in endothelial cell behavior through interaction with integrin receptors

although this is not yet fully understood. Some studies have noted that CCN2 regulates vascular cell growth both by regulating VEGF activity and via inherent structural activities promoting endothelial cell adhesion, migration and survival (Brigstock 2003; Shimo et al. 1999). The observation that CCN2 did not seem to be essential in the embryonic development of the vasculature can simply be explained by a functional compensation by CCN1.

In addition to their extensive interactions with integrins, CCN1 and CCN2 may serve as “docking” proteins functioning as a hub for various receptors and ECM proteins. CCN1 and CCN1 primary sequence contains several binding sites for ECM proteins such as the collagens, fibronectin, decorin, biglycan and vitronectin and although these matricellular proteins do not subserve a structural role in the extracellular environment, their accumulation may affect the mechanical properties of the matrix and alter cell-cell and cell matrix communication. Indeed, the constitutive ECM proteins are natural ligands for several integrin subtypes many of which bind CCN1 and CCN2 as well. Thus, the aberrant expression of CCN1 and/or CCN2 may compete and displace integrins from their natural ligands and either weaken or strengthen cell-matrix anchorage, thus altering cell function and behavior. This hypothesis is supported by the observation that accumulation of either CCN1 or CCN2 in the extracellular environment of pericytes induced anoikis, a form of apoptosis by loss of cell-matrix interactions (Liu et al. 2008).

Another manifestation of CCN1 and CCN2 activities is through their interaction with other bioactive proteins such as cytokines, growth factors, and proteases. These bioactive molecules can either be sequestered in the matrix, presented to their appropriate receptors, or in the case of proteases, inhibited by direct binding or cleared from the pericellular environment by formation of protein–enzyme complexes that are recognized by a scavenger receptor (Armstrong and Bornstein 2003; Workman and Sage 2011). In particular, VEGF¹⁶⁴, which is required for pathological angiogenesis and can be sequestered within the ECM, directly interacts with the TSP1 domain of the CCN proteins which hinders VEGF-mediated neovascularization (Inoki et al. 2002). CCN1 and CCN2 can also bind other receptors that do not typically interact with classical ECM proteins, such as the lipoprotein receptor-related proteins (LRPs) (Segarini et al. 2001) and modulate cell adhesion (Gao and Brigstock 2003).

CCN1 and CCN2 signaling targets clusters of genes with a broad spectrum of biological activities, which probably define their specific activities in either neovascular and fibrovascular reactions. Both CCN1 and CCN2 induce vascular cell growth through modulation of Wnt signaling pathway components, a critical pathway for angiogenesis and vascular development. Silencing of Wnt signaling has been found to lead to vascular deficiencies in tissues ranging from the placenta to the gonads (Si et al. 2006). Remarkably, CCN1-induced Wnt-signaling promoted adhesion, migration, and differentiation of hematopoietic stem

cells into endothelial progenitors and intravitreal injection of hematopoietic stem cells engineered to express CCN1 blocked ischemia-induced neovessel growth subsequent to hyperoxic injury in mice (Hasan et al. 2011). Conversely, CCN2 had no effects on endothelial progenitor growth and differentiation processes. CCN2 rather exacerbates neovascularization, at least in part, through increased expression and activity of MMP-2 which drives vascular remodeling through degradation of matrix and non matrix proteins, migration and invasion of endothelial cells and formation of new vascular patterns (Chintala et al. 2012). The neovascular effects of CCN2 can be suppressed by p53 inhibition and/or MMP-2 inhibition. In this context, it is interesting to note that both CCN1 and WISP-1, a CCN protein closely related to CCN1, can inhibit p53-mediated apoptosis through the activation of Akt pathway (Juric et al. 2012). Thus, the molecular activities of CCN1 and CCN2 are, at times, remarkably different and translate in distinct cell behavior although their actions may occur sequentially rather than concurrently.

Furthermore, both CCN1 and CCN2 may localize in the cytoplasmic and/or nuclear compartments of the cells which adds another layer of complexity to their function. Our previous studies demonstrated both extracellular secretion and nuclear localization of CCN1 in vascular smooth muscle cells (Tamura et al. 2001) while Wahab et al. have reported that CCN2 can be transported via endocytosis to the nucleus after binding at the cell surface (Wahab et al. 2001). The nuclear localization CCN1 and CCN2 or at least their truncated forms, is reminiscent of other examples of proteins found in the nucleus despite their lacking a nuclear localization signal. The growing list of such polypeptides includes epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, angiogenin and parathyroid hormone-related peptide (Jans 1994). These proteins are thought to elicit their biological effects in a bi-functional manner: both indirectly through interaction with cell surface receptor linked to conventional signal transduction pathways as well as through direct association with the nuclei to regulate transcription, translation and mRNA transport (Bryant and Stow 2005). This is consistent with the observation by Planque et al. that truncated forms of CCN3, another member of the CCN family, lacking the secretory signal peptide are directed to the nucleus where they exhibit negative transcriptional activity (Planque et al. 2006). However, further studies are needed to determine the biological significance of the intracellular activities of the CCN proteins and their relevance in the pathological context of neovascularization.

Conclusion

Knowledge of the molecular activities of CCN1 and CCN2 is critical to understand the full scope of their impact in vascular diseases of the eye. The complexity of the function of these proteins was not predicted when they were identified decades

ago. In this regard, CCN3 and CCN4, other members of the CCN family, have been recently shown to exhibit anti-proliferative and/or anti-fibrotic activities and thus, they convey opposing effects to CCN1 and/or CCN2 (El Abd et al. 2013; Shimoyama et al. 2010). How all CCN proteins manifest their biological activities in the context of developmental and pathological angiogenesis, whether some members synergize with or antagonize the activity of other members and whether a functional regulatory relationship exists among them are still unanswered questions. A lesson learned from all CCN-related studies performed so far is that their function can only be fully and accurately appreciated from *in vivo* studies in the context of the whole organism. Future research utilizing transgenic models of targeted compounded gene deletions or overexpression will help elucidate the importance of these molecules and their mechanisms of action in the vascular system. Similarly, studies designed to disable specific motifs in the CCN molecules and generate dominant mutants can provide valuable information on, not only the functional importance of the intact CCN molecules but also the interactions among various CCN proteins. In this regard, CCN3-deficient mice exhibited a normal phenotype while mice expressing a mutant CCN3 gene in which the vWC was disabled produced numerous abnormalities commonly associated with CCN1 or CCN2 gene deficiency (Heath et al. 2008). This suggests that while a functional compensation among various CCN proteins possibly occurs *in vivo*, a dominant mutant form of a CCN protein may impact the biological activities of other members of the family although this concept needs to be experimentally demonstrated. Such knowledge will certainly help in designing therapeutic strategies to better curtail the deleterious effects on abnormal vessel formation and function associated with eye diseases.

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