Balanced regulation of the CCN family of matricellular proteins: a novel approach to the prevention and treatment of fibrosis and cancer

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Abstract The CCN family of matricellular signaling proteins is emerging as a unique common link across multiple diseases and organs related to injury and repair. They are now being shown to play a central role in regulating the pathways to the initiation and resolution of normal wound healing and fibrosis in response to multiple forms of injury. Similarly, it is also emerging that they play a key role in regulating the establishment, growth, metastases and tissue regeneration in many forms of cancer via the interaction of cancer cells with the tumor stroma. Evidence has been recently provided that these proteins do not act independently but are co-regulated working in a yin/yang manner to alter the outcome of both normal physiological processes as well as pathology. The purpose of this review is to twofold. First, it will summarize work to date supporting CCN2 as a therapeutic target in the formation and progression of renal, skin, and other organ fibrosis, as well as cancer stroma formation. Second, it will highlight recent evidence for CCN3 as a counter-regulator and a potential therapeutic agent in these diseases with an exciting, novel potential to both treat and then restore tissue homeostasis in those afflicted by these devastating disorders.

Keywords Diabetic nephropathy · Chronic kidney disease · Skin scarring · CCN2/CTGF · Extracellular matrix pathobiology · Biotherapeutics · Nephrogenic systemic sclerosis · Mesangial cells · Podocytes · Tissue homeostasis · Cancer and tumor stroma

Fibrosis as a therapeutic target

Fibrotic disease can affect any organ and is a leading cause of mortality and morbidity (Wynn 2007). Yet there is no single FDA approved drug specifically designed to arrest or reverse fibrosis. The closest perhaps, nintedanib from Boehringer Ingelheim was created as an anticancer drug, but later developed to arrest fibrosis progression. Recently approved, its indication is limited to idiopathic pulmonary fibrosis (IPF) and treatment does not appear to reverse the disease, but its approval is a step forward and substantiates the opportunity for developing treatments targeted at fibrotic disease. While there are currently a number of drugs in development and/or clinical trials this has only recently been the case. The barriers to development of anti-fibrosis drugs in the past include: 1) fibrosis was not historically a target area covered by pharmaceutical companies 2) fibrosis spans multiple organs and tissues and does not fit neatly into a single therapeutic category, 3) fibrosis has causes and pathways to progression that appeared heterogeneous, 4) fibrosis most often progresses slowly, not only requiring long clinical trials, but since most indications currently lack non-invasive endpoints, measurement of progression and drug effectiveness can be difficult. Over the last 5 years or so, progress has been made in all of these areas and there is, in general, a new optimism about the potential to treat the many fibrotic diseases that now go largely untreated, and a substantial number of drug candidates are in clinical trials. This review focuses largely on the recent

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discovery that the matricellular protein CCN3 is a regulator of critical pathways to fibrosis and as such may be key to the development of a “natural-based” therapeutic with the potential to treat or even reverse diverse forms of fibrosis.

Fibrosis, as part of the wound healing response, is a common response to injury in the kidney. One of the most prevalent progressing forms results in great part from the diabetic environment. Diabetes has for some time been regarded as epidemic in developed countries and is now clearly spreading to developing countries (Ritz et al. 1999). With about 25–35 % being affected, diabetic patients make up the largest population of chronic kidney disease (CKD) patients, and also those progressing to end-stage renal disease (ESRD) (Parving and Hovind 2002). Kidney failure, even with dialysis therapy, results in a high level of morbidity and mortality, the long-term prognosis not being different than many forms of cancer (Parving and Hovind 2002). The cost of treating ESRD is one of the highest among western governments, and will be in developing countries as their economies improve (National Institutes of Health, National Institutes of Diabetes & Digestive & Kidney Disease 2011). Research in the 1990s served to establish the key roles of hyperglycemia, intraglomerular hypertension, and an altered cytokine environment as critical interacting factors in the progression of diabetic nephropathy (DN) (Riser et al. 2000a).

CCN2/CTGF is a critical downstream effector molecule in diabetic nephropathy (DN)

The cytokine TGF- β 1 is considered a principal factor in fibrosis and is known to stimulate extracellular matrix (ECM) production and alter matrix turnover. The ECM, made up of structural proteins, specialized proteins and proteoglycans is critical for holding cells together into tissues, but also dictates how they function. Though TGF- β mediated profibrotic responses have been shown to be exclusively initiated via canonical Smad and non-canonical signaling mechanisms (Barnes and Gorin 2011), additional evidence indicates that the matricellular signaling protein CCN2 is a downstream profibrotic mediator of TGF- β (Riser et al. 2000b). However, this paradigm is complicated by observations that CCN2 activates or co-activates TGF- β -mediated profibrotic responses and appears in itself to play a primary role in fibrosis (Riser et al. 2000b).

In 1998 Ito et al., showed an increase in renal mRNA expression of CCN2 in biopsy samples from patients with a variety of renal fibrotic diseases, including DN (Ito et al. 1998). Thereafter, it was soon shown that CCN2 is dramatically upregulated in corresponding animal models of DN, glomerulosclerosis and tubulointerstitial fibrosis in general, and how it is regulated (Riser et al. 2000b) (Gupta et al. 2000). Around that time it was proposed that the measurement of CCN2 expression and protein levels could serve as the basis

for a diagnostic assay. Both glomerular expression of CCN2 mRNA from kidney biopsy and urine CCN2 protein levels were shown to predict and stage early DN (Adler et al. 2002)(Riser et al. 2003) (Tam et al. 2009) (Nguyen et al. 2006). Additional work subsequently showed that determination of plasma CCN2 levels are able to at least stage progression and that antiproteinuric intervention in non-diabetic nephropathy reduces urine, but not plasma, elevation of CCN2 (Slagman et al. 2011). The first proof as a causal factor was shown by modulation of CCN2 using specific anti-sense oligonucleotide delivery. A downregulation of CCN2 attenuates disease progression in several models of CKD and DN (Yokoi et al. 2004) (Guha et al. 2007). Collectively then, CCN2 has been shown to be a central downstream element in progression of DN, chronically upregulated as the result of hemodynamic, metabolic, and genetic alterations. By 2015, CCN2 has become an established, critical, downstream causal factor in DN and also in the fibrosis that affects a variety of organ systems, including cardiac, liver, and skin fibrosis, as well as encapsulating peritoneal sclerosis (EPS) (Paradis et al. 1999) (Li et al. 2006) (Tsoutsman et al. 2013) (Abrahams et al. 2014).

In terms of the mechanism(s), CCN2 acts to initiate and extend fibrosis by directly increasing the production and accumulation of ECM molecules including collagens, and also likely alters cell migration, cell attachment, and appears to drive a conversion of resident mesenchymal cells to activated myofibroblasts, all of these factors being important in fibrosis development (Riser et al. 2010). The glomerulus is the primary filtering unit of the kidney, and in DN one of the characteristic lesions manifests as accumulation of matrix and fibrosis primarily in the mesangium, commonly referred to as glomerulosclerosis. As the disease progresses, fibrosis also involves the extra-glomerular and peritubular interstitium and tubules. Glomerular mesangial cells, which play an important role in the maintenance of normal glomerular structure and function, provide the framework for the correct mechanical tension on vessels and produce various cytokines, growth factors, metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs), all critical in regulating the proper turnover of the surrounding extracellular matrix (ECM) (Riser et al. 2000a). When the equilibrium is upset, as is the case in DN and a number of other renal diseases, the chronic increased accumulation of glomerular ECM results in fibrosis and that crowding produces a narrowing of capillary lumens resulting in reduced blood flow and impaired kidney function. The podocyte, another glomerular cell type, is also critical DN and certain other kidney diseases. Podocytes are visceral epithelial cells that wrap around the glomerular capillaries and form interdigitating foot processes that rest on the basement membrane. Between interdigitating foot processes are filtration slits that retain larger proteins while allowing small molecules such as water, sugar, and salts to pass and eventually

become part of urine. Podocytes are important in the regulation of the glomerular filtration rate (GFR), and CCN2 expression in podocytes is greatly increased in the STZ-induced mouse model of human type I diabetes (Roestenberg et al. 2006). The exact role of CCN2 in podocyte injury and its contribution to DN is not clear at this time. However, Yokoi et al., have shown that the specific overexpression of podocyte CCN2 in a transgenic mouse model worsens DN and induces the further expression of endogenous CCN2 in the mesangium, this supporting its critical role in the glomerulus (Yokoi et al. 2008).

Potential inhibitors of CCN2 as therapeutics

CCN2 belongs to a group known as matricellular signaling proteins. These matricellular proteins are secreted into the extracellular environment, or ECM, but do not play a primary structural role in this location. Instead, they modulate cell function by interacting with cell-surface receptors, proteases, hormones, growth factors and cytokines, and directly with structural matrix proteins such as collagens (Bornstein 2009). They contain binding sites for these ECM structural proteins and cell surface receptors, and may sequester and modulate activities of cytokines and specific growth factors. Their expression is greatly altered during the response to injury, and they are known to play an important role in wound healing and tissue regeneration. Other members include thrombospondins (TSPs), SPARC, periostin, osteopontin and tenascin-C (Bornstein 2009).

CCN2 is one of a unique 6-member family of matricellular signaling proteins in which members demonstrate similarities in their multimodular structure, but in general have different functions. A number of excellent reviews have been written on this subject (Brigstock 2003), (Holbourn et al. 2008), (Jun and Lau 2011). Therefore, only a brief description will be included here. The CCNs contain 4 modules plus a signaling N-terminal peptide. Three of the 4 modules show partial identity with insulin-like growth factor (IGF) binding proteins, Von Willebrand factor (VWF), and thrombospondin 1 (TSP1), whereas the C-terminal module contains a unique cysteine knot structure (Perbal 2001). CCN5/WISP-2 is unique among the family members in that it lacks the C-terminal, cysteine

knot domain. The CCNs interact with a number of ECM proteins and growth factors, and in some cases specific domains have been identified responsible for this interaction (Jun and Lau 2011). CCNs bind and signal through a number of receptors most prominent being several of the integrins, transmembrane receptors that bridge for cell-cell and cell-ECM interactions (Weston et al. 2003). It appears that often the binding of CCNs to co-receptors such as heparin sulfate proteoglycans, the TrkA receptor, or low-density receptor proteins (LRP) are involved and are thought to help provide for unique signaling (Segarini et al. 2001)(Wahab et al. 2005).

Although CCN2 is now an established target in fibrosis, including that in DN, as of yet there are no approved drugs designed to specifically target CCN2. However, at present there appear to be three companies with programs targeting CCN2 in fibrosis and/or cancer (Table 1). The approaches used and the technologies are all different. Fibrogen Inc. has an anti-CCN2 monoclonal antibody (FG-3019) directed at the elimination of excess CCN2 and is in phase 2 clinical trials in idiopathic pulmonary fibrosis and liver fibrosis following hepatitis B infection as well as pancreatic cancer. RXi has a specific RNAi directed at silencing CCN2, and is in phase 2 in hypertrophic scarring. BRL Bio's drug pipeline is based on the ability of the CCN family member, CCN3, to naturally regulate CCN2 as well as its ability to protect cells from injury. BLR-101 is a recombinant CCN3 and is directed at renal indications, including DN, CKD and glomerulonephritis. BLR-102 and 103 are small peptides that mimic specific regions of the CCN3 protein responsible for anti-cancer and/or fibrotic activity. They are all in the preclinical phase of development and are focused on multiple indications in both fibrosis and cancer.

CCN3 as a regulator of CCN2 and potential inhibitor of fibrosis

The therapeutic approaches described above (and depicted in Table 1) based on antibody clearance and RNAi gene silencing are self-intuitive, and not unlike those currently under development with other disease targets. However, what is that rationale for using CCN3 as a base for therapy? For almost two

Table 1 Current fibrosis and cancer treatments directed at CCN2 (CTGF) Now under development

Drug/Therapy	Company	Drug Type	Development Stage	Indications
FG-3019	Fibrogen	Antibody	Phase 2	IPF, Liver fibrosis due to Hep B Infection, Pancreatic cancer
RXI-109	RXi	RNAi	Phase 2a	Hypertrophic scarring (post revision surgery)
BLR-101	BLR Bio	CCN3 rec protein	Preclinical	Renal fibrosis
BLR-102		Small peptide		Pancreatic and other cancers
BLR-103		Small peptide		Diabetic nephropathy/Liver fibrosis
BLR-105		Small peptide		Skin scarring/Basal cell carcinoma

decades CCN3 (known previously as nephroblastoma over expressed gene [nov]), has been known to play a role in embryogenesis and growth modulation in cancer (Perbal 2001), but until very recently it had not been examined as a modulator of fibrosis or renal disease. It was first shown in mesangial cell culture model of diabetic renal fibrosis that CCN3 and CCN2 work in a yang-like manner to regulate/yang-like manner to regulate the fibrosis development (Riser et al. 2009). In this model, it had previously been shown that exposure of mesangial cells to elevated glucose levels or cyclic mechanical stretching force elevates synthesis and activation of TGF- β 1, and expression of TGF- β receptors leading to increased ECM deposition (Riser et al. 1999). Although TGF- β 1 is a potent modulator of this pathway, it is not the sole stimulator of CCN2. In the above model, upon exposure to TGF- β 1, mesangial cells transition from a cuboidal, non-alpha smooth muscle actin (α -SMA) expressing phenotype to an elongated fibroblast-like cell strongly expressing α -SMA. With this change, CCN2 protein heavily deposited in discrete areas of the ECM at the cell membrane (i.e. between cells) is cast off or lost, while at the same time there is a dramatic upregulation of CCN2 mRNA transcription and translation, the latter resulting in heavily deposited new CCN2 throughout the cytoplasm (Riser et al. 2009). This change is mirrored by similar changes in the distribution and relative amount of COL1. However, when the rat mesangial cells are treated either with a recombinant human CCN3 (rhCCN3), or transfected to highly express human CCN3 prior to TGF- β stimulation these fibrotic changes are almost totally blocked, therefore supporting the CCN3 modulation hypothesis (Riser et al. 2009). Interestingly, mesangial cells in unstimulated, control cultures constitutively produce large amounts of CCN3 that is localized at the membrane and throughout the cytoplasm (confirmed by quantitation by ELISA). TGF- β 1, while potentially turning on new synthesis of CCN2 production, acts in an opposite manner on CCN3 baseline production, reducing it to very low levels (Riser et al. 2010).

CCN3 as a therapeutic agent in DN/CKD: proof of principle in vivo

Using a mouse- specific ELISA it was determined that normal mouse plasma contains approximately 25.3 ng/ml $\pm$ 5.0 (mean $\pm$ SE, n = 15) endogenous CCN3 in the form of freely circulating molecule (Riser et al. 2014). When rhCCN3 was added to mouse plasma and incubated for 48 h at 37 C it remained stable, indicating that it is not quickly degraded by circulating blood proteases. Mice injected (IV or IP) with a bolus of rhCCN3 (50 $\times$ total circulating dose) reached peak drug plasma levels at approximately 10 $\times$ the normal circulating levels of mouse CCN3 (Riser et al. 2014). The kidney

developed the greatest organ accumulation as compared to liver or heart (20 $\times$ greater) and IP delivery produced a significantly greater organ and plasma concentration of rhCCN3 compared to IV delivery. These studies also showed that half-life of circulating and kidney bound drug was between 1 and 8 h.

Animal efficacy studies with rhCCN3 were also recently conducted using the BTBR *ob/ob* mouse (Riser et al. 2014). The BTBR *ob/ob* is a recently developed model of insulin resistance and type II diabetes that greatly mirrors human disease development and progression (Hudkins et al. 2010) (Soler et al. 2012). Due to a genetic alteration in the ability to bind leptin, mice demonstrate increased appetite and become obese. Hyperglycemia is present at one month of age, and by 9 weeks there is a limited mesangial cell lysis, mesangial matrix expansion, some podocyte loss, and resultant albuminuria (Hudkins et al. 2010). By 17 weeks all of these elements of damage and fibrosis progress resulting in measured impairment of glomerular filtration. Beyond this period, and up to 26 weeks, progression uniquely mirrors human later stage DN, when these characteristics become even more pronounced with extraglomerular (interstitial) fibrosis reported (Soler et al. 2012). Using this model a CCN3 efficacy study began treatment at 9 weeks, i.e. when DN was established (Riser et al. 2014). Intraperitoneal injections (0.604 and 6.04 μ g/kg rhCCN3) were administered thrice weekly for eight weeks and the experiment terminated at 17 weeks of age. Fig. 1 shows that rhCCN3 treatment dose-dependently reduced kidney cortex mRNA expression of all four key fibrosis markers examined (CCN2, Col 1a2, TGF- β 1, and PAI-1). The highest dose resulted in a complete blockade with the re-establishment of normal healthy control levels. The levels of the control housekeeping marker (18 s rRNA) were not increased with diabetes nor affected by rhCCN3 (not shown). The treatment with rhCCN3 also completely blocked establishment of glomerular fibrosis as determined by altered mesangial expansion (periodic acid Schiff hematoxylin [PASH]) quantified by non-subjective image analysis (based on 5 images per glomeruli and 25 glomeruli per mouse) (Riser et al. 2014). Previous studies have shown that laminin is a key component of the ECM in glomerular mesangium and tubular basement membranes and that increased deposition occurs in a number of renal diseases including diabetic nephropathy, where there is a marked deposition in either or both of these locations (Ha et al. 1999) (Barnes and Gorin 2011). In the rhCCN3 efficacy studies there was a clear increase in laminin with DN, and the treatment completely lowered its glomerular protein deposition to levels not qualitatively or quantitatively different from those in healthy control mice (Fig. 2). This was mirrored by a concomitant change in the levels of renal cortex CCN2 protein (Riser et al. 2014). Further, the treatment protected against, and even reversed, podocyte loss (Fig. 3) and the establishment of glomerular hypertrophy, key changes characteristic of human

Fig. 1 Treatment with rhCCN3 restores to baseline mRNA levels of key fibrosis genes upregulated in renal cortex by diabetes and obesity. Shown are the levels of mRNA (by RT-PCR) in kidney cortex for 4 key pro-

fibrotic genes. A housekeeping gene as used to assure equal loading, and was unchanged among groups

DN. Functional analysis using clinical parameters also demonstrated the presence of renal impairment (as determined by a significant rise in plasma creatinine) and renal damage (as documented by a significant elevation in the urine albumin to creatinine ratio). In both cases this change was returned to normal levels (Riser et al. 2014). Since both of these

parameters were already elevated at 9 weeks, i.e. the start of treatment, this demonstrated a capacity of the treatment to reverse the established disease.

In summary, the current data supports the postulate (as diagramed in Fig. 4). The general emerging paradigm following renal injury and its progression to a complete wound

Fig. 2 Laminin deposition, a key indicator of fibrosis, is blocked/restored by rhCCN3. Shown in the panel on the left **a-d** is immunostaining for laminin, in renal cortex. Laminin deposition (dark brown color) was limited primarily to the glomerulus in non-obese/diabetic control mice (**a**) Laminin deposition was greatly increased in the diabetic, placebo treated animals (**b**) reduced following low dose rhCCN3 treatment (**c**) and normalized, or near normalized, following the high dose rhCCN3 treatment (**d**). Quantitative results from multiple sections and animals are also shown in the bar graph (right)

Fig. 3 Diabetes and obesity-induced loss of glomerular podocytes is blocked/reversed by rhCCN3 treatment. Shown is immunoperoxidase staining for the p57 podocyte marker, and examined for podocyte injury in renal cortical sections determined by a reduction in cell number (a, b). Quantitative results are shown from the analysis of multiple sections and animals (c)

healing event or, in the case of DN, to marked fibrosis and eventual organ failure is depicted inside the lower large box. In largely non-proliferative fibrosis such DN, glomerular mesangial cells and podocytes experience the insult early (proximal tubule cells and interstitial myofibroblasts are later affected). Increased TGF- β 1 production and activity by these cells is an early response to injury and results in a downregulation of CCN3. This reduced CCN3 allows for the increased transcription and translation of CCN2 mRNA and also its

increased activity. The increase in CCN2 signaling then drives a phenotypic change in mesangial cells to a more myofibroblastic cell type concomitant with an increase in metabolism and deposition of ECM, including COL1. The insult also results in increased expression of podocyte CCN2 along with loss in cell number and function. Once CCN2 is elevated, the level is maintained even in the absence of elevated TGF- β activity (e.g. by autoinduction). Other elements such as abnormal stretching force (changing the adhesive tension of cells to

Fig. 4 Therapeutic model for CCN3 regulation of fibrosis progression and resolution of diabetic nephropathy. Details are described in the text

the ECM) can also increase CCN2 activity in the absence of TGF- β (Riser et al. 2000b). With acute, limited injury, CCN3 levels are soon restored to high baseline, this driving CCN2 production and activity back down with a re-establishment of normal ECM production and complete wound healing or tissue regeneration. However, with a chronic insult (e.g. in DN or CKD) CCN3 levels may actually increase to some degree but remain insufficiently high to block the effects of continually elevated CCN2 (Riser et al. 2010), and thus its downstream alteration in ECM metabolism and its upstream injury to podocytes. The continuous injury and pathway is likely to similarly affect interstitial proximal tubule cells and interstitial fibroblasts. This chain of events allows fibrosis establishment and further progression that, if left unchecked, will progress to the complete loss of organ function (shown in lower right of fig. 4). As opposing molecules in fibrosis development, the CCN3/CCN2 ratio at the site of insult is critical; thereby providing an opportunity for intervention with the administration of rCCN3, or CCN3 mimic peptides (depicted at the top of Fig. 4, and the dotted red arrows). By increasing the CCN3/CCN2 ratio, and therefore adjusting the balance, one can drive the pathway to complete wound healing, ultimately with reversal of the fibrotic lesion and re-establishment of tissue homeostasis (red bold arrow). As shown, this CCN3-driven resolution of fibrosis is through at least two pathways. The first pathway is the protection of podocytes (and likely other renal cells) from injury and loss, allowing cell restoration. The second path is the downstream blockade of altered CCN2 action and the restoration of healthy ECM.

CCN3 in renal proliferative disease

Disease associated with the fibrosis of DN and CKD is regarded as primarily “non-proliferative” and without a large inflammatory component, as are many other pathologies of the kidney. However, there are forms of renal disease that are highly proliferative in nature, with a substantial inflammatory phase, including IgA nephropathy, post-infectious glomerulonephritis (GN), membranoproliferative nephritis and rapidly progressing GN. It is important to understand the potential role for CCN3/CCN2 in these pathologies and their resolution, as patients have little therapeutic option. Van Roeyen et al., using the anti Thy-1 rat model of mesangial proliferative disease showed that CCN3 expression increased after cell proliferation (van Roeyen et al. 2008). The CCN3 peak was commensurate in time with the fall of MC proliferation and appeared driven by upregulation of platelet-derived growth factor (PDGF)-B, since in cell culture PDGF-B suppressed MC CCN3 expression and induced proliferation of MC. Ito, et al. have shown in the anti- Thy-1 model of transient proliferative nephritis that CCN2 mRNA expression is induced very early in the disease in extra-capillary and

mesangial proliferative lesions and then subsides by 14 days when the lesions begin to resolve (Ito et al. 2001). Although they did not examine CCN3 expression in this study, the fall in CCN2 level and resolution was near the same period that Roeyen et al. described the rise and later peak of CCN3. Therefore, collectively these findings support the general yin/yang-like effects of CCN2/CCN3 in proliferative fibrosis, as indicated in this case by the important role for PDGF-B.

CCN3-based therapeutics in skin fibrosis

Development of fibrotic lesions in the skin is a (relatively) common occurrence. Scarring is the manifestation of a healed wound. The scar may be almost undetectable or prominent as in a keloid. Scleroderma (often referred to as progressive systemic sclerosis – though it is not always progressive) is a systemic fibrotic disease with skin manifestation. Other conditions in which fibrotic skin changes are seen include amyloidosis, carcinoid tumors, Dupuytren’s contracture, graft versus host disease, lichen sclerosis/lichen myxedematosus, polymyositis and scleromyxedema (among others). As is the case with fibrosis in other tissues, the major manifestation is the formation of dense connective tissue bundles, consisting largely of type I collagen (COL1). Signaling through the TGF- β pathway is assumed to be largely responsible for this. In spite of its common occurrence, the underlying causes, triggering events and pathogenesis of the various skin fibrotic diseases are not always clear.

Another disease in which fibrotic changes in the skin are prominent is nephrogenic systemic fibrosis (NSF). This is a rare but life-threatening disease that is seen in subjects with compromised kidney function who have undergone a magnetic resonance imaging (MRI) procedure with one or more of the gadolinium-containing contrast agents (Cowper et al. 2000) (Khurana et al. 2007) (Grobner and Prischl 2007) (Swartz et al. 2003). The disease is characterized by the development of hard, “woody” plaques in the arms and legs that may progress up to the trunk. Contractures develop in some cases, and the skin can become discolored and flaky. Although NSF is a true fibrotic disease, lesion histopathology reveals an extensive fibroplasia with mucinous deposits; a picture more similar to that of scleromyxedema than scleroderma (Cowper et al. 2000) (Swartz et al. 2003) (McNeill and Barr 2002). Many of the features seen in patients with NSF have been duplicated in animals (Sieber et al. 2008) (Wagner et al. 2012) (Do et al. 2014).

In vitro studies have shed light on the mechanisms underlying gadolinium-triggered events. Fig. 5 briefly summarizes our current understanding. Two cell types critical to fibrosis – i.e., the fibroblast-like cell, including fibrocytes from the circulation (Quan et al. 2004) (Ortonne et al. 2004) (Wagner et al. 2012) and the monocyte are critical; both respond to

Fig. 5 Model for gadolinium induced fibrosis. Direct effects on fibroblastic cells and indirect effects mediated via monocytes increase the numbers of collagen producing cells in lesional skin, increased procollagen production and modulation of the major enzyme/enzyme inhibitor pathway that regulate collagen turnover

challenge with chelated gadolinium compounds. Fibroblast responses include proliferation, production of matrix metalloproteinase-1 (MMP-1, collagenase-1), production of tissue inhibitor of metalloproteinases-1 (TIMP-1), hyaluronic acid elaboration (Varani et al. 2009) (Bhagavathula et al. 2009) (Bhagavathula et al. 2010) and fibronectin expression (Do et al. 2014). Although the increased elaboration of MMP-1 would seem (at face value) to be anti-fibrotic, the corresponding increase in TIMP-1 assures tight control of MMP-1 function (Perone et al. 2010). Of interest, fibroblasts do not produce type I procollagen in response to gadolinium challenge. This has been shown in isolated fibroblasts and in organ-cultured skin (Varani et al. 2009) (DaSilva et al. 2010). While a direct stimulation of collagen production is not seen in the fibroblastic cell, monocytes respond to gadolinium by elaborating a number of cytokines that can drive TGF-β up-regulation in the skin and collagen production (Wermuth et al. 2009). Perhaps of interest, a similar set of events is thought to underlie pro-fibrotic changes in scleromyxedema (Cokonis Georgakis et al. 2006) (Rongioletti et al. 2010). Taken together, these in vitro studies provide a plausible pathway leading from gadolinium exposure (and retention) to disease. Additional or alternative pathophysiological events may also contribute. For example, if gadolinium becomes de-chelated in the plasma or tissue, the cationic element can quickly form insoluble carbonate or phosphate salts. These can be phagocytosed by resident cells to trigger fibrotic changes in much the same manner as occurs with other mineral dusts. Fibroblasts can phagocytose the strongly cationic gadolinium salts to trigger proliferation and modulate collagen deposition in much the same manner as summarized here with soluble (chelated) gadolinium (Bleavins et al. 2012).

Based on these observations, it was hypothesized that the fibrotic changes seen in the skin of individuals with NSF reflect a direct contrast agent-induced increase in the number of fibroblast-like cells in the lesional skin, a direct effect on the

enzyme/inhibitor system that regulates collagen turnover and an indirect activation (via monocyte/macrophage involvement) of collagen synthesis. This understanding provided a framework to ask whether CCN3 might have a regulatory role in this process. To address this question, human dermal fibroblasts were exposed to one of the chelated-gadolinium compounds and effects of rhCCN3 on the various biological responses were assessed. As part of the experimental design, fibroblasts were exposed to exogenous TGF-β in order to mimic the participation of monocytes/macrophages in the process. In addition, platelet-derived growth factor (PDGF) was also included as a stimulus since past studies have clearly shown that the gadolinium-induced fibroblast growth response depends on PDGF signaling pathway activation (Bhagavathula et al. 2010). As shown in Fig. 6, added rhCCN3 was able to suppress gadolinium-induced stimulation of fibroblast proliferation and down-regulated the enzyme/inhibitor system involved in collagen turnover. The same responses were also shut down when PDGF was used in place of the chelated metal (not shown). At the same time, CCN3 also blocked the elaboration of type I procollagen in the presence of TGF-β (Fig. 6). These findings establish the capacity of CCN3 to act as a multi-functional antagonist of the pro-fibrotic changes occurring in the context of gadolinium exposure. Whether exogenous CCN3 might be used therapeutically in NSF or, perhaps, in other skin fibrotic diseases has not yet been tested.

Also asked was whether endogenously-produced CCN3 might constitute a natural inhibitor of the same processes. First, assessed was CCN3 production by human fibroblasts and found that, indeed, fibroblasts elaborated high levels of CCN3 under normal growth conditions. Of potentially more interest, it was observed in the same study that the same concentrations of chelated gadolinium that stimulated fibroblast proliferation sharply reduced the amount of CCN3 secreted into the culture medium (Fig. 7). A reduction in specific mRNA was observed in parallel (Riser et al. 2012).

Fig. 6 Exogenous CCN3 treatment blocks the biological functions that lead to increased collagen deposition in fibrotic skin. Shown are the effects of gadolinium, PDGF, and TGF- β 1 on human skin fibroblast cell proliferation (a), MMP-1 production (b), and type I procollagen (c)

In summary, NSF is a form of fibroproliferative/fibrotic disease, with the two key cell types being skin fibroblasts (or fibroblast-like cells) and monocytes/macrophages. Presumably, exposure of skin fibroblast-like cells to gadolinium over

Fig. 7 Gadolinium exposure reveals an inverse relationship between fibroblast growth and CCN3 production. Cells numbers were determined following exposure to gadolinium at the indicated concentrations and expressed as a percent of the control untreated cells. CCN3 protein levels were determined from that released into the culture medium

a wide range of concentrations in the presence of gadolinium-stimulated monocytes/macrophages elicit responses that underlie NSF pathophysiology. CCN3 can interfere with all of these responses when provided exogenously, and endogenous production of this inhibitor is down-regulated in response to the same gadolinium concentrations that stimulate the fibro-proliferative/fibrotic changes (Fig. 8). These findings establish the capacity of CCN3 to act as an exogenous inhibitor of the pro-fibrotic events occurring in the context of gadolinium exposure. Likewise, the close relationship between proliferative responses of fibroblasts to gadolinium and suppression of endogenous CCN3 suggests the potential of CCN3, or CCN3 analogs, as endogenous regulators of fibrotic disease.

CCN3 as a potential therapeutic in other fibrotic diseases

It is emerging that the pathways described for the pro-fibrotic actions of CCN2, and its regulation (anti-fibrotic action) by CCN3, do not appear to be unique to renal and skin fibrosis. For example, CCN2 expression is increased in fibrotic liver in patients and experimental animal models, and the levels of plasma CCN2 are elevated in patients with chronic liver disease, being a particularly strong indicator of fibrosis/advanced disease states in hepatitis C (HCV) (Kovalenko et al. 2009).

It was recently shown by Borkham-Kamphorst et al., in models of experimental hepatic fibrosis, that CCN3 mRNA and protein levels increase significantly, mainly in non-parenchymal cells along the areas of tissue damage and repair (Borkham-Kamphorst et al. 2012b). Localization of CCN3 varied, depending on the method and primary site of insult within the liver. PDGF-B (and D) as well as TGF- β suppressed the expression of CCN3, thus mirroring our findings in fibroblasts when modeling proliferative skin fibrosis. Suppression of CCN3 by siRNA enhanced expression of profibrotic marker proteins in primary hepatic stellate cells and cirrhotic fat storing cell line. CCN3 gene transfer by an adenoviral vector, highly expressing the molecule, however, failed to mitigate fibrosis (Borkham-Kamphorst et al. 2012a).

Studies of human atherosclerosis by Oemar, et al. showed that all advanced atherosclerotic lesions of carotid arteries and femoral arteries tested expressed high levels of both CCN2 mRNA and protein.

Localization was mainly to non-proliferating smooth muscle cells in the plaque lesions with some expression in certain CD-31-positive endothelial cells, and predominantly in areas with marked extracellular matrix accumulation and fibrosis. In contrast, normal arteries showed no detectable CCN2 mRNA or protein (Oemar et al. 1997). Another study confirmed this report and further indicated that CCN2 protein may also contribute to atherogenesis by enhancing monocyte migration into atherosclerotic lesions (Cicha et al. 2005). The

Fig. 8 Therapeutic model for CCN3 regulation of fibrosis progression and resolution in NSF. Details are described in the text

co-expression and regulation of the CCNs in atherosclerosis does not appear to have been studied. However, photochemically-induced thrombosis in mice, a knockout of CCN3 results a 6-fold enhancement of neointimal thickening. CCN3 appeared to inhibit neointimal hyperplasia through modulation of smooth muscle cell growth and migration (Shimoyama et al. 2010). Interestingly, treatment with an anti-TGF-β neutralizing antibody alone had no effect. The authors point to CCN3 as a natural inhibitor of neointimal hyperplasia and suggest its usefulness as a modulator of atherosclerotic vascular disease (Shimoyama et al. 2010).

Last, the tumor stroma while largely ignored for decades is now being elucidated as critical to progression, invasion, metastases, and susceptibility to chemotherapeutic agents. The stroma, whereas largely tumor suppressing initially, eventually promotes growth and invasion with changes that often involve the formation of carcinoma-associated fibroblasts (Bremnes et al. 2011). CCN2 is often growth promoting and its overexpression is associated with a number of tumor types suggesting the control of invasion and metastases may be possible by the regulation of CCN2 (Aguiar et al. 2014). Recently a knockout of fibroblast expression of CCN2 in the tumor stroma was shown to diminish the invasion of melanoma cells in the skin and to decrease metastases from skin to the lungs in an animal model (Hutchenreuther et al. 2015). Regulation of CCN2 by CCN3 in cancer stroma has not yet been reported. However, in certain tumors CCN3 has been shown to have negative growth regulatory properties and is clearly a factor associated with repression of tumor advancement (Perbal 2001). Irvine and McCallum showed that CCN3 is a tumor suppressor gene for human chronic myelogenous leukemia (CML) cells in culture (McCallum et al. 2006). Further, patients responding to the chemotherapeutic agent

imatinib show an increase in CCN3 (McCallum et al. 2006). The same group also showed that CCN3 is an important growth regulator in hematopoiesis and that an abrogation of CCN3 enhances leukemogenesis. It also restores growth regulation and sensitivity to induction of apoptosis and CML cell killing with imatinib (McCallum et al. 2009).

The ability of CCN3 to regulate the expression and function of additional CCN family members is also possible. A review of publications on CCNs showed that CCN1, CCN2 and CCN4 have been reported as mostly pro-angiogenic and pro-tumor growth, whereas CCN3 and CCN5 have been shown to exert a negative effect on cell proliferation (Jun and Lau 2011). It was recently shown that the overexpression of CCN3 in mouse NIH3T3 embryonic-derived fibroblasts resulted in a more modest repression of CCN2 mRNA levels while the levels of collagen type 1, a smooth muscle actin, and CCN4 were prominently repressed (Abd El Kader et al. 2013). This is perhaps not surprising for several reasons. First, in the mesangial cell model previously described, the upregulation of CCN2 protein production by TGF-β was reduced approximately 40 %, whereas collagen type 1 was reduced approximately 80 % (Riser et al. 2009). Also, in the animal efficacy studies in DN, rhCCN3 while completely neutralizing the upregulation of CCN2 mRNA was also observed to have anti-fibrosis activity beyond its downstream effect on CCN2. This supports the idea that CCN3 provides a potent anti-fibrosis effect working on multiple pathways that include regulation of CCN2 and, perhaps in at least some fibrotic diseases, both CCN1 and CCN4. The more direct effect of CCN3 on protection of renal cells from injury in DN and the anti-fibrotic effect occurring with PDGF in skin fibroblasts (Riser et al. 2012) suggest that its therapeutic application may go even beyond its ability to balance the pro-fibrotic action of CCN members.

Summary and future direction

The matricellular proteins, including the CCNs, are now established as critical in the process of wound healing and tissue regeneration. A new role for CCN3 is now emerging, as a natural inhibitor or regulator of fibrosis initiation and progression, and it may be key to tissue restoration. Studies are now elucidating an ability of CCN3 to work at multiple levels and on several pathways. CCN3 can work downstream of cytokines like TGF- β to protect from or to dampen the generation of pro-fibrotic factors like CCN2, PAI-1, and MMPs. This appears to reduce, block and perhaps restore the altered deposition of ECM (including collagen type 1) that characterizes diabetic renal fibrosis and many other fibrotic diseases. A critical part of this mechanism of action is its ability to block CCN2 transcription and translation as well as CCN2 binding and signaling. CCN3 can also exert its protective effect by working upstream to protect critical cells like podocytes from injury, the generation of both inflammatory and pro-proliferative molecules including TGF- β and PDGF respectively, as well as ultimately the death of the cell. This activity is observed, and important in fibrosis that is regarded as largely non-inflammatory and non-proliferative as well as related pathologies where inflammation and/or proliferation are key drivers of initiation and/or progression. Current CCN-directed therapies include CCN2-specific antibodies, CCN2-specific RNAi, and CCN3-based recombinant proteins/peptides. The future potential for CCN-based therapy to balance matricellular signaling and restore tissue homeostasis for the prevention and treatment of multiple diseases including fibrosis and cancer is an exciting one.

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Compliance with ethical standards

Competing Interests B. Riser is the founder and CEO of BLR Bio, a biotechnology company dedicated to the discovery and development of novel agents to prevent, treat, and diagnose fibrosis and cancer. He is an inventor of patents for technology designed to utilize CCN3 and CCN3 derivatives in therapeutic and diagnostic applications. J. Barnes is also a consultant to Probetex, Inc., San Antonio, TX. J. Varani has nothing to disclose.

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