Published in final edited form as:

Adv Exp Med Biol. 2011 ; 691: 595–603. doi:10.1007/978-1-4419-6612-4_63.

The Extracellular Matrix Protein CCN1 Dictates TNF α and FasL Cytotoxicity *In Vivo*

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Abstract

It has long been appreciated that the apoptotic activity of TNF α is context-dependent, and requires inhibition of NF κ B signaling or *de novo* protein synthesis to be manifested in most normal cells in culture. Recent studies have uncovered an unexpected pro-apoptotic synergism between TNF cytokines and the CCN family of extracellular matrix proteins, which are dynamically expressed at sites of injury repair and inflammation. The presence of CCN1, CCN2, or CCN3 allows TNF α to induce apoptosis with high efficacy without perturbation of NF κ B signaling or *de novo* protein synthesis, thus converting TNF α from a proliferation-promoting protein into an apoptotic inducer. CCN proteins also enhance the cytotoxicity of other TNF family cytokines including LT α , FasL, and TRAIL. CCN proteins synergize with TNF cytokines through binding to integrin $\alpha_6\beta_1$ and the heparan sulfate proteoglycan (HSPG) syndecan-4 to induce reactive oxygen species (ROS) accumulation. Knockin mice that express a CCN1 mutant defective for binding $\alpha_6\beta_1$ -HSPG are severely blunted in TNF α - and Fas-mediated apoptosis, indicating that CCN1 is a physiologic regulator of these processes. Thus, CCN proteins in the extracellular matrix microenvironment can provide the contextual cues for the cytotoxicity of TNF α and related cytokines, and profoundly influence their activity.

Keywords

inflammation; apoptosis; TNF; FasL; TRAIL; CYR61; CTGF; NOV

INTRODUCTION

Extensive analysis of the apoptotic activity of TNF α has revealed elegant details in its apoptotic pathway, and expression of TNF α is required for or strongly implicated in apoptosis in certain contexts *in vivo* [1-3]. Yet TNF α by itself is unable to induce apoptosis in normal cells in culture, but requires the blockade of *de novo* protein synthesis or NF κ B signaling to be cytotoxic [4,5]. How might TNF α induce apoptosis *in vivo*? Here we present evidence that the extracellular matrix (ECM) microenvironment, specifically the presence of the CCN family of matricellular proteins, can dictate the cytotoxicity of TNF α and related cytokines.

The CCN family of extracellular matrix proteins

The CCN family of ECM proteins consists of six structurally conserved members in vertebrates, and is named after the first three members identified: Cyr61 (cysteine rich 61, CCN1), connective tissue growth factor (CTGF, CCN2), and nephroblastoma overexpressed

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(Nov, CCN3)[6-8]. CCN proteins share a similar modular structure, characterized by an N-terminal secretory peptide followed by structural domains with homologies to insulin-like growth factor binding proteins (IGFBP), von Willebrand factor type C repeat (vWC), thrombospondin type I repeat (TSR), and a C-terminal domain (CT) that contains a “cysteine knot” motif found in some growth factors (Fig. 1). These dynamically expressed proteins regulate diverse aspects of cellular behavior, including cell adhesion, migration, proliferation, survival, and differentiation in many cell types. As such, CCNs are recognized as “matricellular” proteins to draw distinction from classical ECM proteins whose primary roles are critical for the structural integrity of tissues [9,10]. Like other ECM cell adhesion molecules, CCNs function through direct binding to integrin receptors, utilizing cell surface heparan sulfate proteoglycans (HSPGs) as co-receptors in some contexts [6].

Accumulating evidence indicate that CCN proteins regulate the development of the cardiovascular and skeletal systems during embryogenesis, and participate in wound healing and tissue repair in adults [6-8]. Correspondingly, targeted disruptions of *Ccn1* and *Ccn2* in mice result in embryonic and peri-natal lethality due to cardiovascular and skeletal defects, respectively [11-13], although *Ccn3*-null mice are viable and exhibit only modest and transient skeletal phenotypes [14]. In adults, CCNs are highly induced at sites of injury repair and inflammation where TNF cytokines are often co-expressed, providing the opportunity for their interaction.

CCN proteins and TNF cytokines synergize to induce apoptosis *in vitro* and *in vivo*

TNF α is a potent activator of the NF κ B pathway, which drives the expression of many pro-inflammatory and anti-apoptotic genes, thereby promoting cell survival. Paradoxically, TNF α can also activate a powerful apoptotic pathway if NF κ B signaling or *de novo* protein synthesis is blocked [4,5]. Indeed, mice that are deficient in NF κ B signaling die *in utero* from TNF-dependent apoptosis of liver cells [15]. Thus, the apoptotic activity of TNF α is thought to be contextual, subject to sensitizing viral infection or IFN- γ that perturbs NF κ B signaling or protein synthesis. LT α binds the same receptors as TNF α and is thought to act similarly. In contrast, FasL and TRAIL are weak inducers of NF κ B and do not require inhibition of NF κ B signaling to induce apoptosis, but their cytotoxicity is nevertheless regulated by environmental factors.

Our recent studies showed that the extracellular matrix microenvironment, as reflected by the presence of the dynamically expressed CCN proteins, can profoundly regulate TNF cytokine cytotoxicity. The matricellular proteins CCN1, CCN2, or CCN3, either in a soluble form or as cell adhesion substrates, enable TNF α and LT α to induce apoptosis and enhance the cytotoxic effects of FasL and TRAIL without perturbation of NF κ B signaling or protein synthesis, leading to rapid apoptosis [16-18]. Whereas TNF α and LT α did not induce cell death on their own, each of the three CCN proteins enables them to induce apoptosis in ~25% of cells, more than 2-fold higher than the presence of 10 μ g/ml cycloheximide within 4-6 hrs (Fig. 2), further suggesting that CCNs work through a mechanism distinct from inhibition of protein synthesis. These activities appear unique to CCN proteins and are not found in other ECM proteins tested, including collagen, fibronectin, laminin, and vitronectin [16]. Thus, although TNF α alone promotes cell proliferation in fibroblasts by inducing the expression of PDGF [19], the presence of CCN proteins can unmask its apoptotic activity and turn it into a cytotoxic factor.

Since *Ccn1*- and *Ccn2*-null mice suffer pre- and peri-natal lethality, respectively, these animal models are not suitable for studying the effects of CCNs on TNF cytokine functions *in vivo*. To circumvent this problem, we created knockin mice in which the genomic *Ccn1* was replaced by an allele that encodes a mutant CCN1, DM, which is disrupted in the two $\alpha_6\beta_1$ -HSPG binding sites in the CT domain (Fig. 1), leaving the other integrin binding sites

in CCN1 intact [20,21]. The CCN1-DM mutant protein is unable to synergize with TNF α or FasL to promote fibroblast apoptosis [16,17], but is fully active in mediating integrin $\alpha_v\beta_3$ -dependent angiogenic activities [21]. In contrast to *Ccn1*-null mice, *Ccn1^{dm/dm}* mice are viable, fertile, and without any apparent abnormality [16]. CCN1/TNF α synergism was first tested in *Ccn1^{dm/dm}* mice by a subcutaneous injection of a bolus (50 μ l) of high concentration TNF α (0.5 μ M), which rapidly induced cutaneous apoptosis at the injection site. Consistent with the notion that CCN1 is critical for TNF α cytotoxicity, the number of apoptotic cells in *Ccn1^{dm/dm}* mice was reduced by >60% compared to wild-type mice [16].

We further tested the *Ccn1^{dm/dm}* mice in three different models of toxin-induced hepatitis to examine TNF cytokine-mediated apoptosis *in vivo*. First, intravenous delivery of the plant lectin concanavalin A activates inflammatory cells in the liver, inducing hepatocyte apoptosis in a TNF α -dependent process that is abrogated by neutralizing antibodies against TNF α or genetic ablation of TNFR1 or TNFR2 [22,23]. Second, tail vein injection of the agonistic anti-Fas monoclonal antibody Jo2 activates the Fas receptor and leads to massive hepatocyte apoptosis that is completely Fas-dependent [24]. Finally, intragastric administration of ethanol gavage mimics binge drinking and leads to FasL-mediated hepatocyte apoptosis that is prevented by neutralizing antibodies against FasL [25]. In all three experimental models, *Ccn1^{dm/dm}* mice consistently show >60% reduction in hepatocyte apoptosis compared to wild-type mice [16,17]. These results support the notion that CCN1 is a physiologic regulator of TNF α - and Fas-mediated apoptosis *in vivo*, and that its interaction with $\alpha_6\beta_1$ /HSPGs is critical for this activity. Thus, the extracellular matrix microenvironment, specifically the presence of CCN1, can profoundly affect the cytotoxicity of TNF cytokines. These results do not exclude the participation of other factors such as IFN γ , which can also regulate TNF cytotoxicity in certain contexts [26].

ROS mediates signaling cross-talk between CCNs and TNF α or FasL

Upon TNF α treatment, activated TNFR1 recruits the adaptor protein TRADD through its intracellular death domain, leading to the recruitment of RIP and TRAF2 to form a complex (complex I) that is critical for activation of NF κ B and the stress kinase JNK. This signaling complex subsequently dissociates from TNFR1 and recruits FADD and procaspases-8/10 to form complex II, which triggers the activation of the caspase cascade and apoptosis [2,5]. However, the anti-apoptotic c-FLIP, which exists in the cell and is also induced by NF κ B, can compete with procaspases-8/10 for binding to complex II and thereby prevent caspase activation by TNF α [27,28]. Since JNK can phosphorylate the ubiquitin ligase ITCH, which targets c-FLIP for proteasome degradation, a sustained level of activated JNK leads to elimination of c-FLIP and allows the apoptotic pathway to proceed [29]. However, TNF α -activated JNK in normal cells is rapidly inactivated by MAPK phosphatases, which are also induced by NF κ B [30], thus protecting c-FLIP from JNK-mediated degradation. MAPK phosphatases are, in turn, sensitive to the redox state in the cell, and are inactivated by cellular reactive oxygen species (ROS) through oxidation of the critical cysteine residues in their active sites [31]. NF κ B also induces anti-oxidant proteins, including Mn⁺⁺-superoxide dismutase and ferritin heavy chain, which reduce cellular ROS levels [32,33]. Thus, NF κ B suppresses the apoptotic activities of TNF α in normal cells by inducing *de novo* synthesis of c-FLIP, MAPK phosphatases, and anti-oxidant proteins, all of which serve to limit JNK activity and therefore apoptosis.

CCN proteins cross talk with TNF signaling by inducing a high level of ROS, apparently sufficient to override the anti-apoptotic effects of NF κ B, without inhibiting NF κ B activity [16,17]. CCN1-induced ROS allow TNF α -induced JNK activation to be sustained despite NF κ B function, leading to JNK-dependent apoptosis [16]. ROS are also critical for CCN1/FasL synergism, where they trigger the hyperactivation of p38 MAPK, Bax activation and mitochondrial localization, leading to cytochrome c release and apoptosis [17].

Mechanistically, CCN1 induces ROS generation through binding to integrins $\alpha_v\beta_5$, $\alpha_6\beta_1$, and the HSPG syndecan-4 [16], leading to activation of the small GTPase RAC1 [34]. RAC1 regulates ROS generation through multiple mechanisms, including 5-lipoxygenase (5-LOX), certain isoforms of NADPH oxidase (NOX), and the mitochondria [35,36]. In CCN1/TNF α synergism, RAC1-dependent ROS generated through 5-LOX and mitochondria are critical [16]. Although TNF α induces ROS generation through a NOX-dependent mechanism [16,37], this pathway of ROS generation is not required for CCN/TNF α synergism [16]. CCN1 also induces neutral sphingomyelinase 1 (nSMase1) activity [17], which leads to the production of the lipid second messenger ceramide and the generation of ROS [38]. Studies using chemical inhibitor and siRNA showed that nSMase1-dependent generation of ROS is critical for p38 hyperactivation in CCN1/FasL synergism [17]. Thus, CCN1 is able to induce high level of ROS through multiple pathways that override the antioxidant effects of NF κ B.

Future Prospects

Our recent studies unravel a novel mechanism by which the tissue microenvironment directs or reinforces the apoptotic activity of TNF cytokines. In what biological contexts is this pro-apoptotic CCN/TNF synergism important? We have shown that CCN1 is critical for TNF α and FasL-mediated apoptosis in the liver, suggesting that CCNs may participate in hepatocyte injury in a diverse array of liver diseases in which TNF cytokines play a role. For example, TNF α and FasL levels are elevated in hepatic diseases in which apoptosis is induced by toxin exposure, alcohol abuse, and microbial infection. Accordingly, inhibition of TNF α and FasL by means of neutralizing antibodies, soluble decoy receptors, or genetic disruption resulted in reduction of hepatocyte cell death [22,25,39-45]. TNF α and FasL are also critical in cardiomyocyte apoptosis after cardiac ischemia and infarction, conditions where overexpression of CCN1 and CCN2 has been observed, suggesting that CCN/TNF apoptotic synergism may be important in cardiac injury as well [46-50]. Beyond apoptosis, CCNs may also regulate other aspects of TNF cytokine function. These possibilities are currently under investigation.

Acknowledgments

This work was supported by grants from the NIH (CA46565, GM78492, and HL81390) to L.F.L.

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Figure 1. A schematic diagram of CCN1 protein structure

At the N-terminus (N) of CCN1 is a secretory signal peptide, followed by four modular domains with sequence homologies to insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C repeat (vWC), thrombospondin type 1 repeat (TSR), and a C-terminal domain (CT) that contains a cysteine knot motif. All other CCN proteins share the same domain structure except CCN5, which lacks the CT domain. The receptor binding sites identified in CCN1 are: V2, an $\alpha_v\beta_3$ integrin binding site in the vWC domain [51]; T1, an $\alpha_6\beta_1$ integrin binding site in the TSR domain [52]; and H1 and H2, binding sites for $\alpha_6\beta_1$ and HSPGs in the CT domain [20]. The CCN1-DM mutant protein is disrupted in the H1 and H2 sites, thereby abrogating $\alpha_6\beta_1$ -HSPGs-dependent activities while preserving $\alpha_v\beta_3$ -dependent angiogenic functions [21].

Figure 2. Apoptotic synergism between the CCN and TNF protein families

Normal human skin fibroblasts were serum-starved overnight before being treated for 6 hours at 37°C with serum-free media containing purified recombinant CCN1, CCN2, or CCN3 proteins (5 µg/ml each), with or without TNFα (10 ng/ml), LTα (10 ng/ml), FasL (50 ng/ml), or TRAIL (20 ng/ml). Where indicated, cycloheximide (CHX, 10 µg/ml) was added to cells 15 min. prior to addition of TNFα. Apoptotic cells were scored by DAPI-staining as described [16].

Figure 3. Pro-apoptotic signaling cross-talk between CCN1 and TNF α and FasL

The matricellular protein CCN1 binds to integrins $\alpha_v\beta_5$, and $\alpha_6\beta_1$, and the HSPG syndecan-4 to generate a high level of ROS through several mechanisms involving nSMases, 5-LOX, and the mitochondria [16,17]. The level of CCN1-induced ROS is sufficient to override the anti-apoptotic effects of NF κ B by enhancing and maintaining the activation of JNK, which targets c-FLIP for degradation and allows activation of caspases-8/10, leading to apoptosis. In the presence of FasL, CCN1-induced ROS allows the hyperactivation of p38MAPK, which promotes Bax activation and cytochrome c release.